DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

21/2016

NEHA SHRESTHA

Mesoporous Silicon Systems for Oral Protein/Peptide-Based Diabetes Mellitus Therapy

DIVISION OF PHARMACEUTICAL CHEMISTRY AND TECHNOLOGY
FACULTY OF PHARMACY
DOCTORAL PROGRAMME IN DRUG RESEARCH
UNIVERSITY OF HELSINKI

Department of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

Mesoporous silicon systems for oral protein/peptide-based diabetes mellitus therapy

by

Neha Shrestha

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 1, Infocenter Korona (Viikinkaari 11, Helsinki), on 1st April 2016, at 12:00 noon.

Helsinki 2016

Supervisors

Docent Hélder A. Santos
Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

Professor and Dean Jouni Hirvonen
Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

Reviewers

Professor Carla Caramella
Department of Pharmaceutical Chemistry
Faculty of Pharmacy
University of Pavia
Italy

Professor António J. Ribeiro
Faculty of Pharmacy
University of Coimbra
Portugal

Opponent

Associate Professor Alejandro Sosnik
Department of Materials Science and Engineering
Technion – Israel Institute of Technology
Israel

ISBN 978-951-51-2039-7 (paperback)
ISBN 978-951-51-2040-3 (PDF)
ISSN 2342-3161 (print)
ISSN 2342-317X (online)

Helsinki University Printing House
Helsinki 2016

Abstract

Regardless of the considerable efforts, there have been no major breakthroughs in the development of effective oral protein/peptide delivery. When compared to parenteral administration, oral delivery can significantly improve the patients' quality of life, especially in chronic conditions, such as diabetes mellitus (DM), which requires multiple injections daily. However, oral absorption of proteins/peptides is severely limited by their physico-chemical properties and various physiological barriers in the gastrointestinal tract.

Porous silicon (PSi) has emerged as a promising drug delivery system, owing to its beneficial properties, such as top-down production, customizable particle and pore morphology, easy surface modification, simple drug loading, biodegradability and biocompatibility. Thus, the aim of this dissertation was to develop a multifunctional PSi based platforms that would be able to overcome the physiological barriers and efficiently deliver insulin and glucagon-like peptide-1 (GLP-1) orally.

First, the influence of different PSi surface chemistries was evaluated on the intestinal transport of insulin. Due to the negatively charged surface of PSi, there was minimal interactions with the intestinal cells. Thus, chitosan, a polycationic mucoadhesive biopolymer with permeation enhancing effect, was used to modify the surface of the PSi microparticles. When comparing different surface modification techniques, chemical conjugation of chitosan to PSi exhibited strongest cellular interaction, and the highest insulin permeation and uptake across the intestinal cell monolayers. Secondly, three different nanoparticles (NPs) were developed based on lipids, polymers and PSi, with and without chitosan coating, and evaluated as potential oral GLP-1 delivery system. The results showed that the chitosan-modified PSi NPs were the most efficient nanosystem with the best loading degree and the highest GLP-1 permeation across the cellular monolayer.

To overcome several physiological barriers, the next step was to develop a multistage nanocomposite comprising of chitosan-conjugated PSi NPs that were coated with a pH responsive polymer, in order to deliver GLP-1 and dipeptidylpeptidase-4 (DPP4) inhibitor simultaneously via the oral route. This multistage nanosystem showed enhanced GLP-1 transport across the intestinal cell monolayers and across the rat intestinal tissue. Furthermore, the nanosystem also demonstrated hypoglycemic effect *in vivo* after the oral administration in diabetic rats. The efficacy of the nanosystem could be attributed to the combined effect of the permeation enhancing chitosan-modified PSi NPs, the presence of DPP4 inhibitor that prevented GLP-1 degradation, and the pH responsive coating that helped in avoiding premature GLP-1 release/degradation in the stomach.

Moreover, it was shown that the mucoadhesivity and permeation enhancing ability of chitosan-modified PSi NPs could be significantly increased by further surface modification of NPs with either L-cysteine or cell-penetrating peptide (CPP). It was disclosed that electrostatic interactions between the NPs and the glycocalyx were the most prominent pathway for the transport and uptake of insulin from the NPs, together with the contribution of active transport, adsorptive endocytosis and clathrin-mediated endocytosis.

Overall, advanced PSi-based systems were developed which successfully overcame several limitations associated with the oral delivery of biomacromolecules, and thus, showed high clinical potential as oral protein/peptide delivery systems for DM therapy.

Acknowledgements

This study was performed at the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, during the years 2013–2016. These three years of my doctoral studies have been the most exciting, stimulating and challenging time for me, and I would like to express my deepest gratitude for everyone who have helped me during my journey.

I gratefully acknowledge all the funding sources that made my PhD work possible. I was funded by the Finnish Centre of International Mobility (Finland), Finnish Cultural Foundation, Biocentrum Helsinki, and University of Helsinki Research Funds.

I would like to extend my sincere gratitude to my supervisor, Docent Dr. Hélder A. Santos, for his guidance, patience and encouragement that has helped me grow as a scientist. I would also like to show my earnest appreciation for his never-ending support and positive attitude for my scientific endeavours, and also for always pushing me forward. If it was not for him, I would not have been able to accomplish as much as I have.

I would also like to sincerely thank my supervisor Prof. Jouni Hirvonen, for giving me this opportunity to join his group to perform my doctoral studies. I am extremely grateful for his unwavering scientific support, patience and motivation. I also deeply appreciate his kindness and encouraging guidance.

A special acknowledgement to all my co-authors for their invaluable help, sincere cooperation, and productive discussions. I would like to particularly thank Docent Jarno Salonen, Ermei Mäkilä, Prof. Bruno Sarmento, and Dr. Janne Raula for their valuable collaborations and their expertise, positive and creative inputs and discussions.

I would also like to thank Prof. Carla Caramella, University of Pavia (Italy), and Prof. António J. Ribeiro, University of Coimbra (Portugal), who reviewed this dissertation and presented constructive suggestions to improve it.

I would also like to extend my deepest gratitude to my friends Mohammad-Ali Shahbazi, Francisca Araújo, Alexandra Correia, Barbara Herranz-Blanco, Patrícia Figueiredo, Mónica Ferreira and Patrick Almeida for their friendship, support, and thoughtfulness, and for all the wonderful memories we made. Thank you for the countless coffee breaks, cakes, lunches, dinners and all other random events. I am also grateful to all my colleagues at the Division of Pharmaceutical Chemistry and Technology for their good company, pleasant working atmosphere, and unreserved knowledge sharing and technical support.

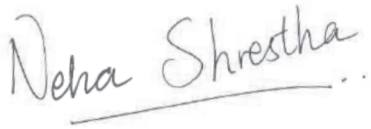
I wish to thank my friends Gaurav, Nitin, Prianca, Sumit S., Prachann, Nitesh, Nischal, Jeevan, Sumit K., Nrupa, Linn, Lisa, Titti and all my friends, old and new, for loving and supporting me and reminding me of the important things in life.

I would like to express my deepest gratification to my parents, for their unconditional love and support. Thank you for always believing in me and encouraging me in my every pursuit. This achievement is yours as much as it is mine. I would also like to thank my brother Shisher, my sister-in-law Siwani, my parents-in-law and my entire family for their love and blessings.

Last but not the least, I want to thank my husband, Bishal, for his unconditional love, constant encouragements, patience and support. Thank you for always being there for me,

in good and bad times. You're the pillar of my strength and nothing would have been possible without you. I cannot thank you enough, ever.

Helsinki, March 2016



Neha Shrestha

Contents

Abstract i
Acknowledgements iii
Contents v
List of original publications vii
Abbreviations viii
1 Introduction 1
2 Review of the literature 3
2.1 Diabetes mellitus (DM) 3
2.2 Aetiological classification of DM 3
2.2.1 Type 1 DM 3
2.2.2 Type 2 DM 3
2.2.3 Other types of DM 4
2.3 DM treatment 4
2.3.1 Insulin therapy 4
2.3.2 Incretin therapy 6
2.4 Oral protein and peptide delivery for DM therapy 8
2.5 Barriers to oral protein and peptide delivery 9
2.5.1 The physical barrier 9
2.5.2 The mucosal barrier 11
2.5.3 The biochemical barrier 12
2.6 Intestinal drug transport mechanisms 12
2.7 Strategies for enhancing the oral absorption of proteins and peptides 13
2.7.1 Absorption enhancers 14
2.7.2 Enzyme inhibitors 14
2.7.3 Increasing the GI retention time 15
2.7.4 Targeting at the site of absorption 16
2.7.5 Particulate-based carrier systems 16
2.8 Porous silicon (PSi) 16
2.8.1 Fabrication and surface modification of PSi 17
2.8.2 Drug delivery applications of PSi-based particulate systems 18
2.8.2 PSi for protein and peptide delivery 20
2.9 Colloidal nanoparticles for oral protein and peptide delivery 22
2.9.1 Polymeric nanoparticles 22
2.9.2 Lipid-based nanoparticles 23
2.10 Biofunctionalization of the carrier systems 24
2.10.1 Chitosan 24
2.10.2 Thiolated chitosan 27
2.10.3 Cell-penetrating peptides (CPPs) 27
2.10.4 Enteric coating of nanoparticles 28
3 Aims of the study 30
4 Experimental 31
4.1 Preparation of micro- and nanoparticles 31
4.1.1 Fabrication of PSi micro- and nanoparticles (I–V) 31
4.1.2 Fabrication of polymer- and lipid-based nanocarriers (II) 32
4.2 Surface modification of the particles 32
4.2.1 Chitosan modification of PSi microparticles (I) 32

4.2.2 Chitosan modification of polymeric, lipidic and PSi nanoparticles (II) 32
4.2.3 Chitosan, L-cysteine and CPP modification of PSi nanoparticles (III–V) 33
4.2.4 Alexa Fluor 488 modification of PSi nanoparticles (III–V) 33
4.2.5 Coating by AFR technology (III–IV) 33
4.3 Physicochemical characterization 34
4.4 Drug loading and release 34
4.4.1 Insulin loading and release from PSi microparticles (I) and nanoparticles (V) 34
4.4.2 GLP-1 loading and release studies from polymeric, lipidic and PSi nanoparticles (II) 35
4.4.3 GLP-1 and DPP4 inhibitor loading and release from polymer-coated PSi nanoparticles (III–IV) 35
4.5 *In vitro* cell based studies 36
4.5.1 Cell lines and cell culturing (I–III, and V) 36
4.5.2 Cytotoxicity 36
4.5.3 Cell–particle interactions 37
4.5.4 Cellular intestinal permeability 38
4.5.5 Cellular intestinal uptake and transport mechanism (I and V) 38
4.6 *Ex vivo* permeability and intestinal adhesion studies (IV) 39
4.7 *In vivo* study (IV) 40
4.7.1 Animal model 40
4.7.2 *In vivo* evaluation after oral administration of the nanoparticles 40
5 Results and discussion 41
5.1 Influence of surface chemistry and chitosan modification of PSi microparticles (I) 41
5.1.1 Insulin permeability and uptake across intestinal cell monolayers 41
5.1.2 Interaction of PSi microparticles with the intestinal cells 43
5.2 Chitosan-modified nanoparticles as oral carriers for GLP-1 (II) 44
5.2.1 GLP-1 encapsulation and *in vitro* release behavior 44
5.2.2 Intestinal transport of GLP-1 from chitosan-coated nanoparticles 45
5.3 Multistage pH-responsive polymer-PSi delivery systems for GLP-1 and DPP4 inhibitor (III–IV) 47
5.3.1 pH-responsive behavior of the multistage polymer–PSi nanocomposite 47
5.3.2 Cellular viability study 49
5.3.3 Enhanced intestinal permeability of GLP-1 across cell monolayers 50
5.3.4 *Ex vivo* GLP-1 and DPP4 inhibitor permeability 52
5.3.5 *In vivo* effect of the nanocarrier 53
5.4 Influence of L-cysteine and CPP functionalization of chitosan-modified PSi nanoparticles on intestinal permeability of insulin (V) 54
5.4.1 Insulin permeability across the intestinal epithelium 54
5.4.2 Cell–nanoparticle interactions 55
5.4.3 Insulin transport and cellular uptake mechanisms 56
6 Conclusions 58
References 60

List of original publications

This thesis is based on the following publications:

I **Shrestha N.,** Shahbazi M.A., Araújo F., Zhang H., Mäkilä E. M., Kauppila J., Sarmento B., Salonen J.J., Hirvonen J.T., Santos, H. A. Chitosan-modified porous silicon microparticles for enhanced permeability of insulin across intestinal cell monolayers. Biomaterials, 2014. 35(25): 7172–7179.

II. Araújo F., **Shrestha N.,** Shahbazi M.A., Fonte P., Mäkilä E., Salonen J., Hirvonen J. T., Granja P. L., Santos H. A., Sarmento, B. The impact of nanoparticles on the mucosal translocation and transport of GLP-1 across the intestinal epithelium. Biomaterials, 2014. 35(33): 9199–9207.

III. **Shrestha N.,** Shahbazi M.-A., Araújo F., Mäkilä E. M., Raula J., I. Kauppinen E., Salonen J., Sarmento B., Hirvonen J., Santos, H. A. Multistage pH-responsive mucoadhesive nanocarriers prepared by aerosol flow reactor technology: A controlled dual protein-drug delivery system. Biomaterials, 2015. 68: 9–20.

IV. **Shrestha N.,** Araújo F., Shahbazi M.-A., Mäkilä E., Gomes M. J., Airavaara M., Kauppinen E.I., Raula J., Salonen J., Hirvonen J., Sarmento B., Santos H. A. Enhanced permeability of GLP-1 based nanocomposites in intestinal tissues and hypoglycemic effect in diabetic animal model. (Submitted)

V. **Shrestha N.,** Araújo F., Shahbazi M.A., Mäkilä E. M., Gomes M.J., Herranz-Blanco B., Lindgren R., Granroth S., Kukk E., Salonen J., Hirvonen J., Sarmento B., Santos H. A. Thiolation and cell-penetrating peptide surface functionalization of porous silicon nanoparticles for oral delivery of insulin. Advanced Functional Materials, 2016. (Accepted).

The publications are referred to in the text by their respective roman numerals (**I–V**). The papers (**I–III**) are reprinted with the kind permission of Elsevier and the paper **V** is reprinted with kind permission of John Wiley & Sons, Inc.

Abbreviations

AF488	Alexa Fluor 488
AFR	Aerosol Flow Reactor
AnnTHCPSi	Annealed thermally hydrocarbonized porous silicon
AnnUnTHCPSi	Annealed undecylenic acid modified thermally hydrocarbonized porous silicon
ATP	Adenosine tyrosine phosphatase
CLDNs	Claudins
CPP	Cell penetrating peptide
CPP-CSUn	Cell penetrating peptide modified chitosan-conjugated undecylenic acid modified thermally hydrocarbonized porous silicon
CSUn	Chitosan-conjugated undecylenic acid modified thermally hydrocarbonized porous silicon
CYS-CSUn	L-cysteine modified chitosan-conjugated undecylenic acid modified thermally hydrocarbonized porous silicon
DAPI	4', 6-diamidino-2-phenylindole, dihydrochloride
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DPP4	Dipeptidylpeptidase-4
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Enhancement ratio
FaSSIF	Fasted state simulated intestinal fluid
FITC	Fluorescein isothiocyanate
GDM	Gestational diabetes mellitus
GI	Gastrointestinal
GIP	Gastric inhibitory peptide/ Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
HBSS	Hank's balanced salt solution
H-CSUn	Hydroxypropyl methylcellulose acetate succinate MF coated CSUn
H-CSUn (D)	H-CSUN loaded with GLP-1 and DPP4 inhibitor
HEPES	2-(4-(2-Hydroxyethyl) piperazin-1-yl) ethanesulfonic acid
HF	Hydrofluoric acid
HPLC	High performance liquid chromatography
HPMCP	Hydroxypropyl methylcellulose pthalate
HPMCAS	Hydroxypropyl methylcellulose acetate succinate
i.p.	Intraperitoneal
kDa	Kilo Dalton
LD50	Lethal dose 50%

MES	2-(N-morpholino) ethanesulfonic acid
NHS	N-hydroxysuccinimide
NPs	Nanoparticles
P_{app}	Apparent permeability
PEG	Polyethylene glycol
PGA	Poly-(γ-glutamic acid)
pI	Isoelectric point
PLGA	Poly(lactic-co-glycolic acid)
PLGA + CS	Chitosan-coated PLGA
PSi	Porous Silicon
Pt	Platinum
PVA	Poly(vinyl alcohol)
PYY	Peptide YY
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SEM	Standard error of mean / Scanning electron microscopy
SGF	Simulated gastric fluid
Si	Silicon
SLNs	Solid lipid nanoparticles
SLN + CS	Chitosan-coated SLNs
TAT	Trans-activator of transcription
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
THCPSI	Thermally hydrocarbonized porous silicon
TJ	Tight junction
TOPSi	Thermally oxidized porous silicon
UnTHCPSi/UnPSi	Undecylenic acid modified thermally hydrocarbonized porous silicon
UnTHCPSi + CS	Chitosan-coated UnTHCPSi
WGA	Wheat germ agglutinin
ZO	Zonula Occludens

1 Introduction

In the last decades, the popularity of proteins and peptides as therapeutic agents have greatly increased, mainly because of their high specificity, potency and effectiveness [1]. This popularity of therapeutic proteins and peptides is further facilitated by advancements in the field of biotechnology and analytical techniques that have allowed large-scale production of several proteins and peptides with high purity [2]. Currently, the market for such drugs is growing steadily with more than 200 biopharmaceuticals approved in both the United States and European Union [3]. However, due to the instability and insufficient bioavailability of proteins and peptides, most of these biomacromolecules are only administered via the parenteral route [4]. Although the parenteral administration leads to high bioavailability, the pain and trauma caused can greatly deter the patient compliance. Furthermore, injectable products have higher manufacturing costs due to the sterility requirements [4, 5]. Thus, several alternative non-invasive routes have been explored for delivering the proteins and peptides, such as pulmonary, nasal, buccal, rectal, ocular, transdermal and oral [4, 5]. The need of non-invasive delivery systems is even more evident for diabetes mellitus (DM), due to the chronic nature of the disease and the need of two or more injections of insulin per day for effective glycemic control in T1DM patients [6]. Insulin and glucagon-like peptide-1 (GLP-1) are the proteins/peptides used in DM therapy. In DM, the use of oral route can have additional benefits for insulin and GLP-1 delivery, as it more closely mimics the endogenous physiological pathway, thus allowing better glucose homeostasis [7-9].

Regardless of several benefits of oral delivery of proteins and peptides, the poor oral bioavailability (usually less than 1–2%) severely limits their therapeutic outcome [5, 10]. There are several factors that act together to restrict the oral absorption, which must be carefully considered while developing efficient oral protein and peptide delivery systems. These factors include the physicochemical properties of proteins/peptides (e.g., molecular weight, stability, hydrophilicity, molecular size) and the physiological barriers in the gastrointestinal (GI) tract (e.g., variable pH conditions, enzymatic degradation, semipermeable mucus layer and tight junctions between the intestinal epithelial cells) [5, 10, 11]. Despite the considerable amount of time and efforts invested to develop innovative approaches which could overcome the limitations of oral protein and peptide delivery, an effective system still remains unaccomplished [10].

There are various strategies to enhance the bioavailability of proteins and peptides after oral administration. One of the strategies includes the modification of the protein/peptide structure to impart hydrophobicity or to make the compound stable against the enzymatic degradation [12]. However, such modifications could compromise the biological activity of the proteins/peptides [12]. Another popular approach explored is the formulation approach, such as the use of permeation enhancers, mucoadhesive compounds, and micro- and nanosystems [13-15]. The particulate-based drug delivery systems have several benefits, such as modifiable surface properties to acquire tailored cellular interactions, dual drug delivery for synergistic therapeutic outcome, pH-responsivity that allows protection from the harsh gastric conditions, as well as site specific controlled drug release [16]. A number

of drug delivery systems have been studied for the oral delivery of proteins/peptides, such as polymer, lipid, and porous silicon (PSi)-based micro- and nanosystems [17-20].

Among several drug delivery systems, the PSi based system has shown great potential as a carrier for proteins and peptides [21-24]. This mesoporous carrier has several advantageous properties, such as custom-made particle and pore morphology, simple surface modification (physical adsorption or chemical conjugation), mild drug loading conditions, incorporation of diverse molecules or NPs, and established biocompatibility and biodegradability [25-27]. Nevertheless, the negatively charged surface of the PSi materials hinders the interaction of the system with the mucus layer and the intestinal epithelia due to the repulsion from the negatively charged mucin glycoproteins. Therefore, chitosan, a polycationic biopolymer would be a beneficial addition to the surface of PSi-based carriers, which would inherit mucoadhesive and permeation enhancing properties to the PSi carrier [28, 29]. Further modification of the chitosan-modified carrier systems with thiolated groups or cell-penetrating peptide (CPP) could further facilitate and improve the particles' interactions with the intestinal epithelia to enhance the transport of the encapsulated biomacromolecules. Thiolated chitosan forms disulfide bonds between the immobilized thiol groups and cysteine-rich subdomains present in the mucus glycoproteins, thereby greatly increasing the mucoadhesion property of the compounds [30]. Likewise, a positively charged CPP has the ability to augment the intracellular transport without causing any damage to the cells [31].

The harsh acidic and enzyme–rich gastric environment can lead to the degradation of the proteins and peptides, resulting in the substantial loss of the expected therapeutic outcome. Consequently, an enteric coating of the carrier is a crucial step to overcome the instability issues [32]. One approach that could be utilized to form polymeric coatings on the PSi particles is aerosol flow reactor (AFR) technique. AFR is a simple single-step process to coat micro- and nanoparticles (NPs) with polymers, and has been previously used to prepare different micro- and nanoparticles successfully [33-35]. The possibility to avoid harsh organic solvents during the coating process is an added benefit for this process. This method allows a close control of the particle properties by easy regulation of the temperature and residence time in the heating chamber [36-38]. Moreover, the polymeric coating also provides an additional site for incorporating another drug, thus providing a possibility for dual-drug delivery [21].

This dissertation focuses on the development of PSi-based micro- and nanosystems for the oral delivery of insulin and GLP-1 for DM therapy. Chitosan was employed to enhance the mucosal and cellular interactions of the developed systems with the intestinal epithelia, along with the enhancement of intestinal permeability of the drugs. Furthermore, thiolation and CPP modification of the systems were performed to augment the permeation enhancement of the encapsulated protein/peptide. The understanding on how the developed PSi-based systems functioned was also acquired by studying the mechanisms of insulin uptake and transport across intestinal cellular monolayer. This work also focuses on developing multifunctional nanosystems by enteric coating, in order to deliver peptides efficiently and successfully via the oral route.

2 Review of the literature

2.1 Diabetes mellitus (DM)

Diabetes mellitus (DM) is a group of metabolic disorders mainly characterized by the high blood glucose levels (hyperglycemia) and disturbances in the metabolism of carbohydrates, fats and proteins [39]. It is mainly caused due to partial or absolute loss of insulin production and/or insulin resistance in target cells [39-41]. According to the International Diabetes Federation, in 2014 there were 387 million individuals suffering from DM worldwide, and this number is expected to rise to 592 million by 2035, making DM one of the biggest health problems in the world with very high socio-economic impact [42]. In Europe alone, ca. 52 million people suffer from diabetes, out of which 33.1% remain undiagnosed [42]. The main symptoms of DM include hyperglycemia with polyuria, polydipsia, weight loss, polyphagia, ketoacidosis and blurred vision. In long term, DM can lead to severe complications, such as hypertension, retinopathy, nephropathy, acute neuropathy, amputation and cardiovascular diseases [39].

2.2 Aetiological classification of DM

2.2.1 Type 1 DM

Type 1 DM (T1DM), also known as insulin-dependent DM or juvenile-onset diabetes, represents 5–10% of the total DM cases [41, 43]. It is characterized by absolute loss of insulin production in the body due to the destruction of insulin producing pancreatic β cells, which is considered to be triggered by immune-associated, if not immune mediated mechanisms [43]. The rate of β cells destruction is rather variable, thereby causing differences in the onset of disease, from as early as childhood (most common) to as late as the ninetieth years [39, 40]. The progressive T1DM shows ketoacidosis as the first indicator, but the slower forms can show mild hyperglycemia, which can rapidly change to a severe state of hyperglycemia or ketoacidosis in the presence of stress or infections [39, 40]. Exogenous insulin administration is required to maintain normoglycemic levels and to prevent the occurrence of side effects, which if uncontrolled can lead to coma and death [41, 44]. The aetiology of the disease is not clearly defined, but it is mostly related to the genetic predisposition and environmental factors [41].

2.2.2 Type 2 DM

Type 2 DM (T2DM), also known as non-insulin dependent DM or adult onset diabetes, is responsible for 90–95% of the DM cases [41]. In T2DM, there is a relative loss of insulin production and/or loss of insulin action. In some cases, high blood glucose levels are

observed with normal or elevated insulin levels and impaired insulin sensitivity. However, in other cases, the hyperglycemia observed is related to relative loss of insulin production with normal insulin action [39, 40]. The symptoms of T2DM are not easily noticeable because of the presence of only mild and gradual hyperglycemia, leading to a large number of undiagnosed cases [39, 41]. Such T2DM patients are at risk of having micro- and macrovascular diseases. The cause of this disorder is not clearly understood. Nevertheless, the risk of T2DM is often associated with age, obesity and sedentary lifestyle. Also, the patients who have been previously diagnosed with gestational DM (GDM), hypertension, dyslipidemia and genetic predisposition (complex and not clearly understood), may develop T2DM [39]. Several pharmacological (oral hypoglycemic agents) and non-pharmacological (*e.g.*, weight loss, diet, and physical activity) treatment approaches are applied alone or in combination to restore normoglycemia [45].

2.2.3 Other types of DM

GDM is another type of DM, which occurs in women during pregnancy. Such women either can continue to have DM or can show normal glucose tolerance after the end of pregnancy. Nonetheless, the risk of subsequently developing T2DM is high in such cases [39, 41]. There are several other types of DM, which will not be dealt here in detail, such as (i) genetic defects of β cell functions, (ii) genetic defects on insulin action, (iii) exocrine pancreas disease, (iv) endocrinopathies, (v) drug or chemical induced, (vi) infection-induced, (vii) uncommon forms of immune-mediated diabetes, and (viii) other genetic syndromes [39, 41].

2.3 DM treatment

The Diabetes Control and Complications Trial and the United Kingdom prospective diabetes study have reported that the glycemic control is the principal way of preventing the occurrence of diabetic complications [46, 47]. DM requires chronic treatment to control the blood glucose levels and prevent the occurrence of both acute and long-term life-threatening complications associated with it. In addition, the type of pharmacotherapy also depends on the underlying pathological condition.

2.3.1 Insulin therapy

Insulin is the most versatile and effective therapy used to control blood glucose levels in DM patients. Replacement therapy with insulin involves its exogenous administration by subcutaneous injections, multiple times a day [48]. For the patients diagnosed with T1DM, hormonal replacement therapy with insulin is inevitable. These patients only require small doses of insulin to maintain glucose homeostasis, as there is no loss of insulin sensitivity, despite the absolute loss of insulin production. Conversely, for T2DM patients, due to the

poor insulin sensitivity, higher doses of insulin are required with gradual dose increase [49]. Nevertheless, the occurrence of hypoglycemic events is one of the major side effects of insulin therapy [50].

Since the discovery of insulin by Banting and Best in 1921, there has been great progress towards the production, purification and structural modification of insulin to achieve different pharmacokinetic profiles [51]. Insulin is a heterodimeric polypeptide divided into two chains, A and B, linked together by three disulfide bonds as shown in **Figure 1** [51]. A human preproinsulin consists of 110 amino acid residues and comprises of A-chain (21 amino acids), B-chain (30 amino acids), C-peptide (31 amino acids) and signal peptide (24 amino acids). The preproinsulin is cleaved to obtain the mature insulin peptide, consisting of only the A- and B-chains [51].

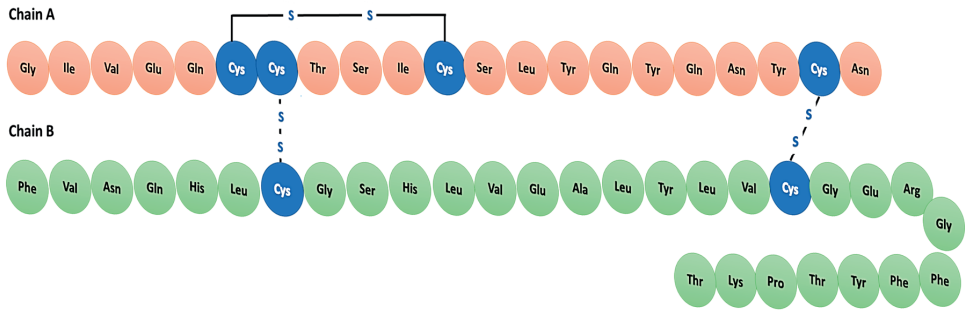


Figure 1 *Primary structure of human insulin.*

Insulin is produced by the β cells of islets of Langerhans in pancreas and its release is stimulated by glucose in a concentration dependent-manner [51]. Insulin release is a very dynamic and complex process, and it occurs in biphasic pattern with a first rapid phase that lasts only few min, followed by a sustained phase [52, 53]. Insulin acts on different tissues (*e.g.*, liver, adipose tissues, muscles and brain), where it influences the glucose uptake, glycogenesis, glycogenolysis, and glycolysis. In addition, insulin is also involved in carbohydrate, lipid and protein metabolism [51].

Insulin analogues are mainly characterized based on the onset of action, duration of action and peak effect. The rapid acting insulin analogues have an onset of action of 15 minutes with peak at ~1 h. Insulin Glulisine (Apidra®), Insulin Lispro (Humalog®) and Insulin Aspart (Novolog®) are the different types of rapid-acting insulin. The regular or short-acting insulin (*e.g.*, Humulin® R and Novolin® R) is effective for 3–6 h with its peak at ~2–3 h. The intermediate acting insulin, Insulin NPH (*e.g.*, Humulin® N and Novolin® N), is effective for a period of ~12–18 h. The long acting insulin tends to lower the blood glucose level over a 24 h period. Insulin Detemir (Levemir®) and Insulin Glargine (Lantus®) are the two types of long-acting insulin [54, 55].

2.3.2 Incretin therapy

The treatment regimen for T2DM patients is more complex, and usually includes life style modification and/or drugs, as the first line of treatment. The selection of the oral hypoglycemic agents used, with or without insulin, depends on several considerations, such as drug interactions, side effects, costs and drug efficacy [45]. The drugs used for the treatment of T2DM are sulfonylureas (stimulate insulin secretion), biguanides (suppress hepatic glucose secretion), α-glucosidase inhibitors (delay digestion and intestinal absorption of carbohydrates) or thiazolidone and metformin (improve insulin sensitivity and peripheral glucose uptake) [56]. Despite being widely used, these treatment approaches fail to address the continuous loss of β cell function with time and weight gain associated with the T2DM [56].

In the past decades, the use of incretin hormones in DM therapy has emerged as a potential treatment approach, as evidence of the role of incretin polypeptides in post-prandial glycemic control have been reported [57, 58]. La Barre, in 1932, first coined the term "*incretin effect"*, referring to the gut hormones that stimulate insulin secretion after nutrient ingestion [57-59]. The first incretin hormone to be discovered was the gastric inhibitor peptide (GIP), also known as glucose-dependent insulinotropic polypeptide. It is a 42 amino acid peptide that is secreted from the K cells present on the proximal small intestine [60]. However, it was realized later that GIP was not responsible for the full incretin effect, revealing the presence of another incretin factor released from the distal region of the bowel [61, 62]. Subsequently, the second incretin hormone, GLP-1, was discovered in 1987 [63, 64]. GLP-1 is synthesized from the enteroendocrinal L cells present in the distal ileum and colonic mucosa as a 37 amino acid peptide GLP-1 (1–37), which is cleaved into two circulating forms of GLP-1 (7–37) and (7–36) [63, 65]. Although both the forms have equally potent insulinotropic effect, GLP-1 (7–36) is more abundant [65]. The primary structure of GLP-1 (7–36) is shown in **Figure 2**. The secretion of both incretin hormones is stimulated by ingestion of carbohydrates and lipids containing diet [62], and thus secreted incretin hormones act on the G-protein coupled receptors in pancreatic β cells, resulting in the enhancement of the release of insulin containing granules [66].

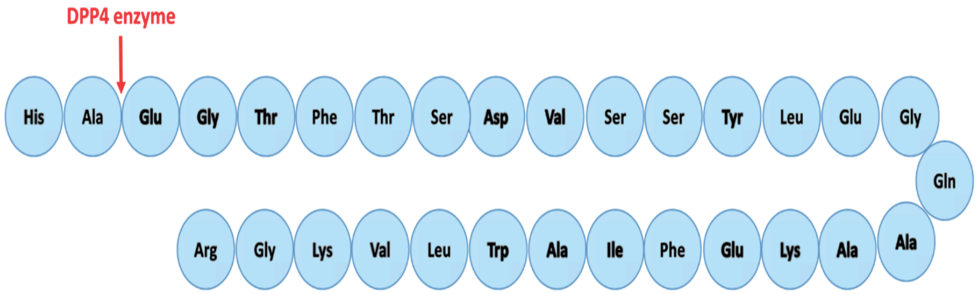


Figure 2 *Primary structure of GLP-1 (7–36), indicating the position where dipeptidyl peptidase (DPP4) enzyme cleaves the peptide to yield the inactive form of the molecule.*

In 1986, Nauck and colleagues showed that patients who suffered from T2DM had deficient incretin effect [67]. The deficient incretin effect was due to the defective GIP action and reduced GLP-1 secretion, thus, establishing only GLP-1 as a potential clinical target for T2DM therapy [67-69]. One of the main advantages of GLP-1 as an antidiabetic agent is that its effect is lost when the blood glucose concentration is lower than 77 mg/dL, thereby reducing the risk of unwanted hypoglycemic events [70, 71]. Moreover, GLP-1 also has several other actions, such as (i) up-regulation of insulin biosynthesis, (ii) enhanced insulin gene expression, (iii) increased β cell mass, (iv) inhibition of glucagon secretion, and (v) delaying of gastric emptying and suppression of food intake (as shown **Figure 3**) [59, 62]. All of these favorable actions establish GLP-1 a highly promising antidiabetic agent. GLP-1 also affects other peripheral tissues, such as brain, heart and liver, as shown in **Figure 3** [59, 72]. However, the bioactive GLP-1 needs to be administered by continuous infusion as the peptide has a very short half-life of 2 min, owing to rapid degradation by the proteolytic enzyme, dipeptidyl peptidase-4 (DPP4) [73, 74].

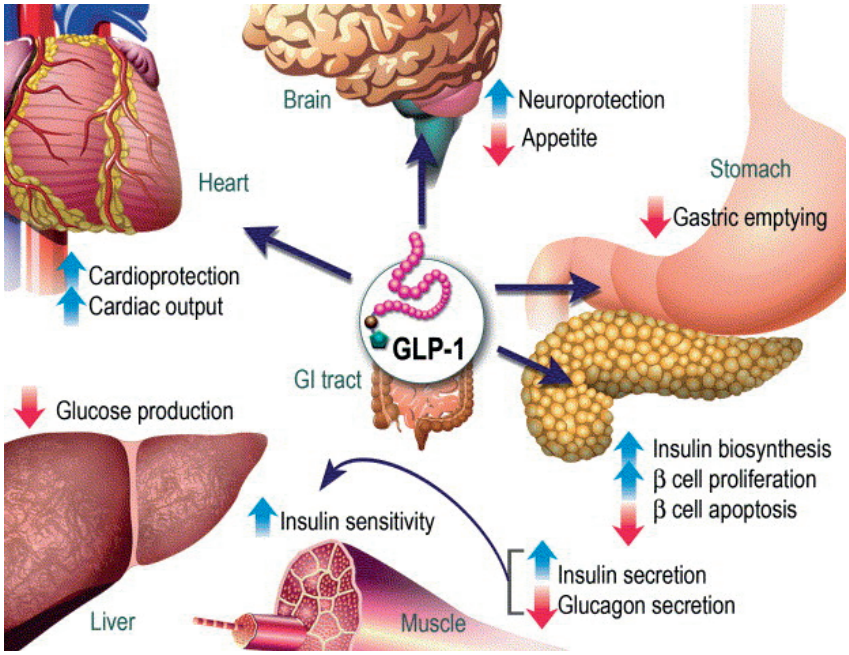


Figure 3 *The action of GLP-1 on several peripheral tissues. GLP-1 acts directly on endocrine pancreas, heart, stomach and brain, and indirectly on liver and muscle [72].*

DPP4, a CD26 serine protease, deactivates peptides and proteins by cleaving them at the amine terminal which has alanine or proline amino acids in position 2, as shown in the structure in **Figure 2** [75]. The DPP4 enzyme is abundantly available in both membrane-bound forms (liver, kidney, lungs, intestine, pancreas, spleen, adrenal gland, and the central nervous system) and as soluble protein in the circulation [59, 73]. Since, DPP4 enzyme degrades and inactivates GLP-1, it has also been established as a potential clinical target

[76]. Studies have shown that, in human subjects with T2DM, when the DPP4 activity was inhibited for 4–52 weeks, potentiation of β cell function, enhanced insulin sensitivity and suppression of plasma glucagon were observed, which could be beneficial for the treatment of T2DM [77-79]. In addition, selective DPP4 inhibitors have shown to be comparatively safe in high doses in mice, rats, and dogs [80].

There are several DPP4 inhibitors, such as vildagliptin (Novartis) and sitagliptin (Merck), which efficiently inactivates the DPP4 enzyme and, consequently, increases the GLP-1 levels. A potent and selective DPP4 inhibitor, NVP-DPP728, which potentiates insulin release in response to glucose-containing meals, has also been reported [81]. The inhibitor is described to have an excellent oral bioavailability and potent antihyperglycemic effect [82]. The long-term inhibition of DPP4 enzyme with NVP-DPP728 showed improvement in the glucose tolerance in both normal and glucose-intolerant mice through improved islet function, increased insulin secretion, and protection from increased islet size in insulin resistance, as well as with improved prandial glucose homeostasis [83, 84].

2.4 Oral protein and peptide delivery for DM therapy

Oral protein and peptide delivery as a non-invasive alternative approach is still one of the biggest challenges for formulation scientists, with oral insulin delivery being the holy grail of DM treatment. The delivery of such biomacromolecules as an injectable formulation has several drawbacks associated with it, such as the poor patient compliance caused by the fear, pain and physiological trauma associated with injections [2]. In DM therapy in particular, despite the satisfactory performance of the current parenteral insulin therapies, there are numerous adverse effects associated with it, such as hypoglycemic episodes, infections at the injection sites, peripheral hyperinsulinemia, local hypertrophy due to the insulin deposition at the site of injection, insulin edema, and atherosclerosis due to the irregular insulin absorption [7]. The need of life-long insulin therapy with more than 60,000 injections throughout the patients' lifetime is a major limitation [40, 85]. In addition, the cost-ineffectiveness, need for proper storage conditions, and high risk associated with the use of parenteral route are other limiting factors for this administration route [86].

Consequently, a number of alternative non-invasive delivery routes for proteins and peptides have been studied, such as oral, pulmonary, buccal, nasal, ocular, transdermal and rectal [26]. Among them, the oral route is the most popular one because of its accessibility and close resemblance to the physiological pathways of both insulin and GLP-1 [7-10]. Furthermore, the extensively used subcutaneous administration of insulin leads to direct entry in the peripheral circulation, exposing the peripheral tissues to abnormally high amounts of insulin, thereby causing peripheral hyperinsulinemia [7, 87]. Additionally, in contrast to the parenteral route, the oral route also has other advantages, such as high patient compliance, ease of administration and relatively low production cost [10, 88].

A number of pharmaceutical companies have also invested in developing a viable oral delivery system for DM therapy, with some of them already in clinical stages. **Table 1** summarizes some of the efforts made by industry on developing oral formulations for insulin and GLP-1, and their current development status.

Table 1 *Examples of oral protein and peptide delivery systems for DM therapy under clinical trials.*

Product	Technology Description	Status	Reference
Capsulin™ OAD and IR (Diabetology, UK)	Axcess™ delivery technology enteric-coated capsule containing a mixture of insulin, an absorption enhancer, and a solubilizer.	Entering Phase IIb (OAD) and Phase II (IR)	[89]
ORMD-0801 (Oramed, Israel)	A mixture of insulin and adjuvant that protects and promotes intestinal uptake of insulin, filled in an enteric-coated capsule.	Phase II	[90]
I320GT (NN1957) (Novo Nordisk, Denmark)	A long-acting oral basal insulin analogue as once-daily tablet treatment.	Phase I	[91]
Oral HDV Insulin (Diasome, USA)	Hepatic directed liposomal vesicles for oral insulin administration.	Approved for Phase III	[92]
OG987SC (NN9927) (Novo Nordisk, Denmark)	A once-daily tablet treatment of a long-acting oral GLP-1 analogue.	Phase I	[91]
ORMD-0901 (Oramed, Israel)	Oral GLP-1 capsule based on the company's POD™ technology.	Phase I	[90]

2.5 Barriers to oral protein and peptide delivery

The natural course of ingested proteins and peptides present in our diet involves breakdown into small peptide fragments and/or component amino acids, which are then easily absorbed via the intestinal epithelium. However, following the same route, the therapeutic proteins and peptides, like insulin and GLP-1, will completely lose their physiological action after the breakdown. In addition, the absorption of the remaining small fraction of active proteins/peptides is further diminished by their poor permeability across the mucus covered tightly arranged intestinal epithelium [10]. Hence, it is of utmost importance to understand the features and functions of these physical and biochemical barriers of the GI tract to develop an effective oral protein/peptide delivery system.

2.5.1 The physical barrier

The GI epithelium layered with mucus acts as physical barrier for the absorption of proteins and peptides, permitting only selective absorption of nutrients, fluids and electrolytes, whilst avoiding the passage of harmful substances and pathogens [10, 93]. The length of the GI tract is ~6 m, and has high regional variations in regards to pH, mucosal thickness and composition, residence time, enzymatic activity and surface area [32].

The small surface area, harsh acidic environment along with the presence of the pepsin enzyme renders the stomach as an unfeasible site for protein/peptide absorption [10]. In contrast, the human intestinal epithelium is a highly absorptive region with a total surface area of 300–400 m^2. The cells comprising the small intestinal epithelium are categorized

into absorptive enterocytes, secretive goblet, enteroendocrinal and Paneth cells, and M cells (in Peyer's patches), as shown in **Figure 4** [5, 94]. The enterocytes are the most abundant and rapidly renewing epithelial cell lines (life span of 3–4 days) which are derived from the stem cells present at the base of the intestinal crypts [5]. These cells are compactly packed with organized and well-ordered microvilli present on the apical side. The presence of microvilli results in expanded surface area, allowing enhanced nutrient absorption [10].

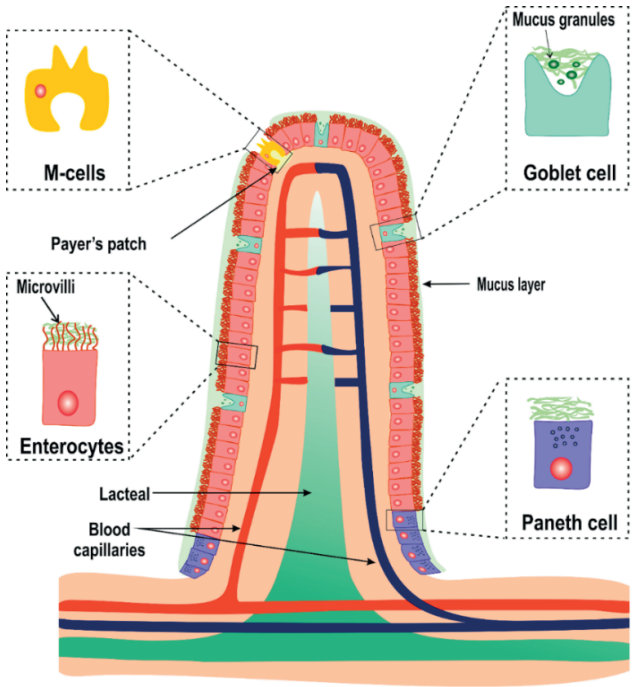


Figure 4 *Schematic representation of the structure of the intestinal epithelium composed of mucus layer, enterocytes, goblet cells, Paneth cells, and M cells in Peyer's patches.*

Goblet cells are the second most abundant cell type in the intestinal epithelium and they are located interspersed between the epithelial cells. These cells also have a lifespan of 3–6 days, and their number increases from the duodenum to the terminal ileum [5, 95]. The third type of cells present in the intestinal epithelium are the Paneth cells which are located at the base of crypts of Lieberkühn and they have the ability to kill bacteria by secreting anti-microbial proteins [95]. Additionally, specialized epithelial cells, named M cells, are present in specialized regions in the intestinal epithelium known as Peyer's patches. These are flattened cells with no mucus layer present on their surfaces [5, 96]. Furthermore, low aminopeptidase activity in the region of Peyer's patches and high endocytic activity of these cells provide a good site for protein/peptide absorption [5, 97]. The intestinal epithelium also consists of hormone secreting enteroendocrinal cells [5, 95]. Besides the small intestine, the colon also acts as a possible absorption site for proteins/peptides, mainly due to the absence of enzymatic activity and prolonged residence time [98].

The physical integrity of the intestinal epithelium can be attributed to the presence of tight junctions (TJs) or *Zonula Occludens* (ZO) in the paracellular space between adjacent epithelial cells [5, 11]. Owing to the presence of TJs, the intestinal epithelium acts as the rate-limiting barrier for paracellular transport, regulating the passage of various components between the intestinal lumen and submucosa. TJs are dynamic structures mainly composed of transmembrane integral proteins (Claudins (CLDNs), occludens and junctional adhesion molecules), intracellular plaque proteins (ZO-1, ZO-2, ZO-3, cingulin, and 7H6) and regulatory proteins [11, 99]. The complex and organized interplay between these components are very important for the assembly of the TJs. The transmembrane proteins are responsible for sealing the adjacent cells, and the intracellular plaque proteins play an important role in giving structural support to the TJs [11, 100]. The regulatory proteins are assumed to be involved in signal transduction and cell adhesion, however, their involvement in the regulation of TJs has not been clearly defined yet [11, 101]. Furthermore, the architecture of actin cytoskeleton has been shown to be vital for the function of TJs, as several actin binding proteins can serve as an anchor to the plasma membrane [101].

2.5.2 The mucosal barrier

The mucus layer covering the intestinal epithelium is one of the limiting factors for the absorption of orally delivered drugs [102]. The mucus barrier functions as a protective barrier for the epithelial cells against the harsh environment, and helps in trapping and clearing pathogens and foreign particles [103, 104]. The barrier is composed of three different layers. The first layer is the membrane-bound mucin, known as glycocalyx, on the top of the epithelial cells. It plays a role in docking the mucus layer to the epithelium and may have a role in mucoadhesion [104, 105]. The mucus produced by the goblet cells blanketing the glycocalyx acts as the second barrier. The last barrier is composed of bicarbonate ions secreted by the epithelial cells [102].

Mucus is a highly hydrated (≥95 % water content) viscoelastic gel lubricating and protecting the different surfaces of the body [103, 106]. It is a complex heterogeneous blend of carbohydrates, proteins, lipids, lysozymes, antibodies, cell debris, bacteria and inorganic salts [105, 107]. Among them, the major components are the large mucin fibers that are entangled and crosslinked with each other by disulfide linkages and hydrophobic interactions, and are mainly secreted by the goblet cells and submucosal glands. The mucin fibers are rich in negatively- charged glycosylated regions and hydrophobic domains [108]. The mucin fibers forms a dense porous structure due to the high degree of crosslinking, which creates steric obstruction [109].

The thickness of the mucus layer across the GI tract varies extensively, with the thickest mucus layer present in the stomach and colon, and the thinnest in the jejunum [93]. There is a continuous turnover of mucus, (intestinal turnover of 50–270 min), where the old layer is shed off and discarded, digested or recycled [109, 110]. The mucus layer offers the first line of defense against several foreign materials that enter the intestine and is an important barrier against the absorption of protein and peptide absorption. Thus, the underlying characteristics of this layer must be clearly understood to overcome it.

2.5.3 The biochemical barrier

Besides the physical barrier, the GI tract also presents a biochemical barrier for oral protein and peptide delivery. This biochemical barrier comprises of the high pH variations spanning the GI tract and the presence of several enzymes, such as pepsin, trypsin, chymotrypsin, aminopeptidase and carboxypeptidase [102, 111]. The site of enzymatic degradation can be the lumen, brush border, cytosol of enterocytes, lysosomes or other cell organelles [112]. The highly acidic environment in the stomach (pH 1.5–3.5) and the presence of proteolytic enzyme, pepsin, induces hydrolytic degradation of the ingested proteins and peptides [102, 112]. Moreover, duodenum and jejunum have high brush border enzymatic activity, which together with other digestive enzymes create harsh conditions for the proteins and peptides [102]. Nonetheless, the Peyer's patches present in the duodenum and ileum have ~20–30 % of the aminopeptidase activity as compared to the nearby regions [97]. In addition to the enzymatic degradation, as the pH rises to ~6 when entering the small intestine, there is a possibility for some of the proteins and peptides to be precipitated, which cannot be easily re-dissolved [102].

2.6 Intestinal drug transport mechanisms

The transport of drugs across the intestinal epithelium can be either active (energy dependent) or passive (non-energy dependent) [113]. Active process involves the transport against the concentration gradient, and requires direct or indirect consumption of adenosine tyrosine phosphatase (ATP) molecules [113]. Passive transport involves the movement of molecules in the direction of a concentration gradient, following the Fick's law of diffusion [10]. The mechanism through which a molecule is transported depends on its physicochemical characteristics, such as molecular weight, chemical structure, pK_a and hydrophilic–lipophilic balance [114]. There are two possible drug transport pathways across the intestinal epithelium: paracellular and transcellular (receptor-mediated and carrier-mediated), as depicted in **Figure 5**.

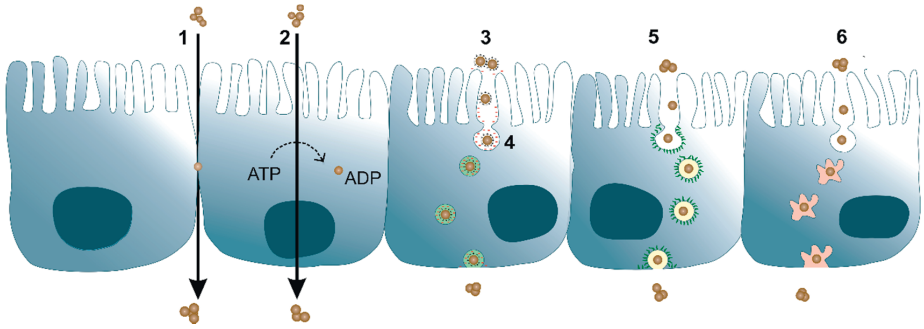


Figure 5 *Schematic representation of different transport mechanisms in the intestine:* ***1.*** *Paracellular pathway;* ***2.*** *Active transport;* ***3.*** *Electrostatic interactions between positively charged NPs and negatively charged glycocalyx* ***4.*** *Adsorptive mediated endocytosis* ***5.*** *Clathrin-mediated endocytosis; and* ***6.*** *Caveolae-mediated endocytosis.*

The paracellular transport involves the passage of molecules through the water-filled intercellular space between two conjoining epithelial cells [10, 102, 114]. In the case of hydrophilic proteins/peptides, the paracellular route is the most preferred transport route, and is determined by their molecular size and ionic charge [115]. However, the convoluted pathway and the presence of TJs (as aforementioned) between the epithelial cells can greatly reduce the possibility of easy passage of molecules [115]. The paracellular space is in the order of 15 Å, which greatly limits the passage of large molecules through this pathway [116]. Nonetheless, it has also been reported that high conformational flexibility of proteins and peptides makes it possible for larger molecules to use this route as well [117]. Since the paracellular space comprises of only 0.01–0.1% of the total intestinal surface area, the amount of drugs transported via this route is limited, thus suggesting the involvement of other routes complementing the paracellular passage [10, 113].

Transcellular drug transport involves the movement from the apical to the basolateral membranes of the epithelial cells [10]. As the cell membranes are composed of lipid bilayers, passive transcellular transport is highly favorable mostly for lipophilic drugs. The active transcellular route involves endocytosis of the molecule, starting at the apical membrane, transport through the cells and release at the basolateral side [118]. Transcellular passage occurs mainly in M cells and the enterocytes. Studies have shown that an intestinal model comprising of M cells had 5-fold higher transport when compared to Caco-2 cell monolayers [119, 120]. Phagocytosis via M cells, micropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis are examples of active transcellular mechanisms [121]. Clathrin-mediated endocytosis, which is able to internalize particles up to 15 nm in size, and phagocytosis, which can engulf micron-sized particles, are receptor-mediated endocytosis mechanisms. Such receptor-mediated mechanisms involve the binding of the ligand to the receptor present on cell's surface and then internalization through coated vesicles [102, 122]. The fate of such mechanism of cellular internalization are highly dependent on the ligand-receptor binding [114, 122].

2.7 Strategies for enhancing the oral absorption of proteins and peptides

There are several approaches that have been studied in order to enhance the oral bioavailability of biomacromolecules and can be broadly classified into two categories: chemical structural modification and formulation based strategies [114]. The structural modification approach involves altering the physicochemical properties of the protein/peptide by modifying its molecular structure, whilst maintaining or enhancing its pharmacological action. The commonly used chemical strategies include prodrug formation, PEGylation, lipidization, site-specific mutagenesis and glycosylation [114, 123]. Nonetheless, these optimization methods are limited by the structural complexity, possible inefficient modifications and additional production costs for the structural modifications [123, 124]. Alternatively, the formulation approach exploits the use of different pharmaceutical excipients and novel drug delivery systems to successfully deliver

proteins/peptides orally, by protecting them against the enzymatic degradation and/or by enhancing the intestinal permeability [10, 114].

The following sub-sections shed some light on the different formulation approaches investigated to deliver proteins/peptides.

2.7.1 Absorption enhancers

Absorption enhancers are used to enhance the intestinal permeability of proteins and peptides, via the paracellular or transcellular route [114, 123]. Bile salts [125], chitosan [28], thiomers [126], cell-penetrating peptide (CPP) [17], medium chain fatty acids [127], chelators [128], and ZO toxins [129] are some examples of the commonly used of absorption enhancers [2, 114, 115, 130]. All these absorption-enhancing agents interact with different components of the intestinal epithelium to increase the transport of drugs. The absorption enhancing mechanisms may include changing of the membrane fluidity, decreasing the mucus viscosity, opening up of TJs, and cell penetration enhancement [2]. However, once the integrity of the intestinal epithelium is compromised either by altering the permeability of the cell membrane or by opening up of the TJs, there is a high possibility for undesired entities (such as pathogens, bacteria and foreign particles) to also enter the systemic circulation, leading to severe complications [131]. Thus, local irritation and long-term toxicity must be considered while using these absorption enhancers. Nonetheless, several absorption enhancers with reversible influence on the intestinal epithelium have also been reported [132].

2.7.2 Enzyme inhibitors

Proteins and peptides are highly susceptible to degradation by several enzymes present in the GI tract. Therefore, inhibiting the degrading enzymes, such as trypsin, chymotrypsin, carboxypeptidases and aminopeptidases, can be one of the approaches to increase the amount of biologically active molecules [10, 114]. The selection of enzyme inhibitors is dependent upon the nature of the protein and the enzymes involved in its metabolism. Long term use or the use of non-specific inhibitors can lead to unwanted toxic effects, and can possibly alter the metabolism of other proteins in our normal diet [15, 114]. There have also been efforts to inhibit the enzymatic action without the use of any inhibitors, by altering the physiological conditions, such as the pH [133].

A number of inhibitors have been studied for enhancing the permeability of insulin, such as aprotinin, bacitracin, soybean trypsin inhibitors, and Bowman–Birk inhibitor [134, 135]. In the case of GLP-1, DPP4 inhibitors are the most commonly used inhibitors to prolong its very short half-life [65].

2.7.3 Increasing the GI retention time

Another important strategy that can be employed for improving the transport of proteins and peptides is increasing their residence time in the GI tract, which can be achieved by mucoadhesion (specific or non-specific) [136]. Besides increasing the residence time, the mucoadhesive systems also allow the biomacromolecules to come in close proximity to the absorption site in the intestine [136]. Chitosan, thiomers, methacrylate, and alginate are some of the most commonly used mucoadhesive agents [137, 138]. Khutoryanskiy categorized these mucoadhesive polymers as anionic, cationic, non-ionic, amphoteric, thiomers, dendrimers, synthetic glycopolymers, etc. [139]. The mechanism of mucoadhesion depends on the features of the polymers used, such as surface charge, chain flexibility, surface tension, hydrophobicity and hydrophilicity [136]. Additionally, for delivery systems composed of such mucoadhesive polymers, the interaction with mucus depends on the different characteristics of the delivery system, such as size, surface area, surface chemistry, and surface charge [105].

There are several theories that have been proposed to explain and understand the mechanisms of mucoadhesion, and they are summarized as follows [139, 140]:

- *Electronic theory* proposes the involvement of electrostatic interactions between the negatively charged mucin with the oppositely charged materials.
- *Adsorption theory* proposes the involvement of hydrogen bonding, van der Waals force, hydrophobic interactions and chemisorption in mucoadhesion.
- *Wetting theory* correlates the surface tension to the mucoadhesion as the ability of the mucoadhesive material, in liquid form, to spread over the mucus layer.
- *Diffusion theory* considers the interpenetration of the mucoadhesive material into the mucus gel, and the diffusion of soluble mucin into the dosage form. This process is driven by the concentration gradient.
- *Fracture theory* considers the difficulty to detach two previously joined surfaces.
- *Mechanical theory* proposes the role of roughness of the surface, which promotes the mucoadhesion due to the increased contact surface area.

Since the process of mucoadhesion is complex in nature, more than one theory needs to be used to explain the given conditions [140].

The adhesion process has two major stages: contact and consolidation. The contact stage is the first step where the mucoadhesive material must come in close contact with the mucus layer. However, the process is complicated for orally delivered systems as it is not possible to directly place the mucoadhesive system close to the mucus layer in the GI tract [140]. Next, the consolidation stage leads to prolonged adhesion due to various physicochemical interactions to strengthen the adhesion. Peppas and Sahlin proposed a theory explaining the process of consolidation, which is based on the diffusion theory, and includes the interpenetration of the mucoadhesive molecules with the mucus glycoproteins and formation of secondary bonds. Thus, indicating the contribution of both surface and diffusional phenomena to form strong interactions between the mucoadhesive polymer and mucus [141].

2.7.4 Targeting at the site of absorption

Due to the large variations along the GI tract, site specific release of proteins/peptides in regions with a thin mucosal layer, maximum uptake and low enzyme activity, can be utilized to obtain enhanced drug absorption [10, 142]. Additionally, the delivery of biomacromolecules can be targeted to release in a specific region of the intestine, small or large, by using pH-sensitive polymers [15]. Another approach can be the use of targeting moieties for site-specific delivery of proteins and peptides [102]. Several targeting moieties, such as lectins, vitamin B12 and peptide ligands (CSKSSDYQ) have been reported to enhance the intestinal absorption of proteins and peptides [2, 102]. In addition, CPPs have also been successfully applied to enhance the intestinal permeability by entering into the cells via endocytic pathway [143]. Some promising results regarding the use of targeted oral protein/peptide delivery have been reported, nonetheless, the possibility of toxic reactions on the chronic use of such targeting ligands need to be further studied in detail.

2.7.5 Particulate-based carrier systems

Particulate-based delivery systems provide an alternative way to deliver proteins/peptides orally. The incorporation of biomacromolecules into the particulate system can offer several advantages, such as protection against the enzymatic degradation and harsh acidic conditions in the stomach, controlled drug release, targeted delivery at the site of absorption and enhanced intestinal absorption [2, 12]. There are different kinds of particulate systems that have been presented as excellent carriers for oral delivery of proteins and peptides. These particulate-based systems could be matrix-based or porous in nature, micro- or nano-sized, composed of polymers, lipids or mesoporous materials, prepared by bottom-up or top-down approaches. Polymeric drug delivery systems (*e.g.*, hydrogels, micro- and nanospheres and NPs), lipid-based drug delivery systems (*e.g.*, micro and nano-emulsions, liposomes and lipid NPs) and mesoporous particles (*e.g.*, silica and silicon-based micro- and nanoparticles) are some examples of the most investigated particulate systems for oral drug delivery [16, 144]. The properties of the particulate carriers, such as size, surface charge, surface chemistry and the presence of targeting ligands, have a great influence on its fate in the GI tract [145]. For example, the uptake of the particulate system highly depends on their size, as the intestinal uptake increases as the size of the system decreases [145, 146].

The ability to incorporate one or more strategies, such as absorption enhancement, enzyme inhibition, mucoadhesivity, targeting and enteric coating into one optimum system is one of the main benefits associated with the particulate systems. In addition, the biocompatibility and biodegradability of the delivery systems are also vital for the delivery of proteins/peptides, especially in the case of DM due to the chronic nature of the disease.

2.8 Porous silicon (PSi)

Porous silicon (PSi) was first reported 60 years ago by Uhlir at Bell laboratories [147], while it was not until 15 years after its first reference that the porous nature of the material was

recognized by Watanabe *et al*. [148]. Prof. Leigh Canham demonstrated the bioactive property of PSi and its ability to impart either bioinert, bioactive or biodegradable nature just by altering the porosity and pore size of the material [149-151]. These findings have led to immense popularity of this material in biomedical applications, both in drug delivery and biosensing [25, 150, 152, 153].

PSi materials have several advantageous properties, such as high surface-to-volume ratio, large surface area and pore volume, high stability (*e.g.*, thermal, chemical and mechanical), biocompatibility and biodegradability [25]. The top-down fabrication process allows easy scaling-up, and the pore and particle size can be easily controlled by altering the fabrication parameters [153-155]. Furthermore, easy surface modification of PSi with different types of biopolymers and targeting moieties also has benefit for biomedical applications [156-158].

2.8.1 Fabrication and surface modification of PSi

The most common PSi fabrication method is electrochemical anodization, using monocrystalline silicon (Si) wafers as a positive anode and platinum (Pt) as a negative cathode [25, 151, 155, 159]. Aqueous or ethanolic hydrofluoric acid (HF) is used as an electrolyte solution. The use of ethanolic solution allows reduction in hydrogen bubble formation and enhances the electrolyte penetration into the pores, to allow uniform PSi layer formation [25]. A schematic representation of an anodization cell used to prepare PSi is shown in **Figure 6** [25].

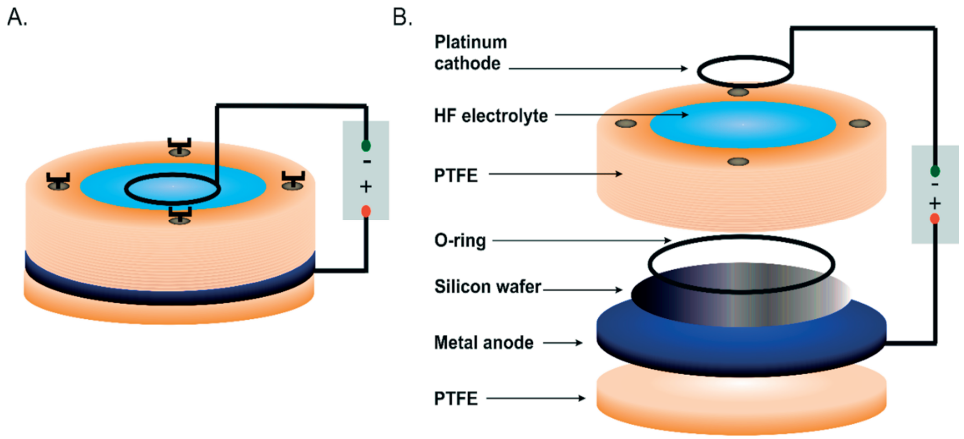


Figure 6 *Schematic illustration of the anodization cell for one side etching of Si-wafer (A) and construction of the cells with different components (B).*

In this arrangement, the Si-wafer is placed in between two electrolyte cells with Pt electrodes on both sides. The front side of the Si-wafer acts as the anode, and the backside of Si, which is in close contact with conductive metal anode, serves as the cathode. When

an etching current is applied between the electrodes, a layer of PSi is formed on the Si surface. The metal deposition in one side of Si allows a homogenous PSi layer formation in the front side [25, 160]. The pore morphology of PSi material can be controlled by altering some of the fabrication parameters, such as HF concentration, electrolyte composition, current density, wafer type and resistivity, crystallographic orientation, temperature and illumination intensity [151, 155, 159]. Some of the other methods used for the PSi fabrication include stain-etching and zero-current etching [161, 162].

The freshly prepared PSi is an unstable hydrogen-terminated form (Si-H_x), which is susceptible to rapid oxidation leading to the loss of its structural and optical properties [25, 159]. Relative humidity, temperature and composition of air have great influence on the rate and extent of oxidation [25, 159]. In drug delivery applications, the use of the native Si-hydride form can lead to the degradation of adsorbed drugs, thereby greatly compromising its applicability [151, 163]. Thus, as most of the applications require stable surface chemistry, the surface of PSi needs to be stabilized prior to use.

The most common methods for stabilization of PSi are oxidation, hydrosilylation, thermal carbonization and thermal hydrocarbonization [25]. Controlled oxidation of the surface of PSi, to form Si–O bonds, imparts hydrophilicity to the PSi, which facilitates the wettability and dissolution in physiological fluids [155, 164]. Thermal, anodic, photo, and chemical methods can be used for the oxidation of the PSi surfaces. Another surface stabilization method is hydrosilylation to yield Si-C surface with high hydrolytic stability [151]. It involves the reaction of alkene or alkyne with Si–H bond, by thermal, photochemical or Lewis-acid catalyzed [25, 155]. Thermal hydrocarbonization of Si is another stabilization method, that is prepared by treating the Si–H surface with acetylene at high temperature (500 °C) to form a highly stable hydrophobic Si–C_xH_y surface [165]. Further thermal treatment of Si–C_xH_y with undecylenic acid allows the addition of –COOH terminated groups to the surface of the PSi layer [22, 166]. The presence of this –COOH layer allows easy covalent attachment with other biopolymers, macromolecules, or fluorescent dyes.

2.8.2 Drug delivery applications of PSi-based particulate systems

The understanding of the biocompatibility and biodegradability of any material is highly important before its application in the field of biomedicine. In the case of PSi, the relationship between bioactivity of the material and its pore morphology has already been established [150]. In addition, it has also been shown that PSi degrades mostly into monomeric silicic acid, which is the most naturally occurring available form of Si in the environment [25]. A number of *in vitro* and *in vivo* studies have been performed to show the biocompatibility of PSi, along with the evaluation of their inflammatory and immune responses [167-172]. Shahbazi *et al*. showed that the toxicity of NPs was predominantly dependent on the surface chemistry and charge of the NPs [171]. Several other studies have also demonstrated the biocompatibility of PSi-based micro- and nanoparticles with GI cell lines [18, 173], heart tissues [172], and human ocular cells [174].

Along with excellent biocompatibility, the unique high surface area-to-volume ratio of the PSi system also makes it a potential candidate for drug delivery applications. In addition,

PSi also possesses unique capability to efficiently load and control the release of drug molecules [26, 151, 153, 175]. The drug loading and release patterns can be controlled by altering the characteristics of PSi materials, such as surface charge, pore size, surface hydrophilicity/hydrophobicity and loading method. Additionally, it is also influenced by the physicochemical properties of the loaded drug [26, 155, 164]. The incorporation of drugs into the pores of PSi can be performed by either immersion, oxidation-induced trapping, impregnation or covalent attachment methods [155]. Out of these loading techniques, the immersion technique is the most commonly used, as it is a simple process and has the possibility to be performed at room temperature. This technique follows the principle of physical adsorption of the drug molecule onto the pore surfaces of PSi. Charge and hydrophobicity, along with the concentration of the drug loading solution, loading time and temperature, are factors that influence the drug adsorption onto PSi [25, 152]. Depending on the physicochemical characteristics of the drug and PSi, different drug release behaviors, from burst to sustained release, can be obtained [157, 175].

PSi particles have been reported to enhance the solubility of very poorly water-soluble drugs [176, 177]. This advantageous property is mainly due to the ability of PSi to confine the drug molecules in its pores in an amorphous-like or nanocrystalline form, which has significantly higher solubility as compared to the crystalline form [154, 178]. Sorafenib [179], celecoxib [180], methotrexate [156, 181], ibuprofen [175], indomethacin [21], itraconazole [182], ethionamide [183], griseofulvin [154, 175] and furosemide [175] are some of the poorly water-soluble drugs, whose dissolution has been greatly enhanced by incorporation in PSi particles.

In addition to the enhanced dissolution profiles of drugs, PSi has been further modified and developed to form advanced multifunctional drug delivery systems. For example, Herranz-Blanco *et al*. developed a hybrid nanocomposite made-up of PSi NPs and stimuli responsive polymeric material, polyethylene glycol-block-poly(L-histidine), using a flow-focusing microfluidic chip [184]. The nanocomposites were able to demonstrate plasma stability, high cytocompatibility, reduced internalization by phagocytic macrophages and cell growth inhibition in a pH-dependent manner [184]. Similarly, Kong *et al.* showed the fabrication of an advanced biocompatible multifunctional nano-in-micro platform consisting of PSi NPs and giant liposomes, which were also assembled in a microfluidic chip [185]. The system was aimed at controlled co-delivery of hydrophilic and hydrophobic drugs along with DNA nanostructures. Additionally, short gold nanorods, and magnetic NPs were also incorporated into the system to allow photothermal and magnetic responsiveness.

The possibility of surface modification of PSi is very useful for its application as a drug delivery system. For example, Sarparanta *et al*. used a gastro-retentive class II hydrophobin protein to modify the surface of PSi NPs, with the aim to increase the gastric mucoadhesion of the NPs [186]. The results showed enhanced biocompatibility and increased transit time of PSi NPs in the stomach, however the mucoadhesive property was lost in the intestine leading to rapid transit of the NPs [186]. In another example, the surface of PSi NPs were decorated with a cell surface binding peptide to target the murine B-lymphoma cells. The targeting moiety used in combination with PSi NPs showed efficient *in vivo* targeting [187]. Similarly, Wang *et al*. employed a simple copper-free click chemistry to modify PSi NPs with targeting peptides, which resulted in enhanced intracellular uptake and tumor specific targeting [157].

PSi particles have also been successfully reported as gene delivery systems for cancer therapy [188, 189]. Additionally, other smaller NPs, such as quantum dots [190], carbon nanotubes [191], hollow gold nanoshells [192], and Fe_3O_4 NPs [193], have also been successfully loaded into the pores of PSi. The incorporation of such NPs into the pores of PSi have added benefits to the delivery system, such as photothermal therapy in the case of hollow gold nanoshells [192], or targeting by magnetic field in the case of Fe_3O_4 NPs [193].

2.8.2 PSi for protein and peptide delivery

Developing an effective formulation for protein and peptide delivery is a complex process and requires consideration of several factors. PSi, as a carrier for proteins and peptides, offers a unique advantage of mild aqueous drug loading conditions and the possibility to avoid the use of harsh organic solvents [155]. Foraker *et al*. reported one of the first account of using PSi particles to encapsulate protein molecules [194]. The study presented the ability of PSi microparticles to enhance the intestinal permeability of insulin across Caco-2 cell monolayers. Since then, there has been several studies demonstrating the successful incorporation of proteins and peptides into the PSi particles, aiming at drug delivery through various routes, such as oral [21, 194, 195] and subcutaneous [22, 196-198]. Electrostatic and hydrophobic interactions are considered the key forces that act during the protein/peptide adsorption to the PSi pores [199, 200].

Several studies have been performed to understand different possible parameters that can influence the adsorption of proteins and peptides into the pores of PSi particles [21, 197, 198, 201]. These factors include the nature of the protein/peptide, pore size, particle size, surface chemistry of PSi, and loading conditions. The relationship between the size of the protein molecule and the pore size plays an important role in determining the loading capacity. Karlsson *et al*. studied the influence of the mean pore size and the thickness of the PSi layer on the loading of human serum albumin [202]. The results showed that the albumin was encapsulated only when the pore radius was larger than 5.5 nm. In addition, it was also shown that when the PSi thickness layer was greater than 1 μm, albumin was not able to occupy the available surface area completely [202]. In another study, Prestidge *et al.* studied the effect of size and hydrophobicity of protein/peptide on loading efficiency in PSi wafers [203]. In this study, two different macromolecules, gramicidin A (~1.8 kDa, hydrophilic) and papain (~23 kDa, hydrophobic) were used. The study showed that the large hydrophobic papain molecule was not efficiently loaded and was instead accumulated on the surface of PSi. However, the smaller hydrophilic peptide, gramicidin A, was readily loaded into the pores of PSi [203]. Similarly, another recent study by Pastor *et al*. showed that the release of proteins could be sustained by decreasing the pore size [204]. In the study, PSi of pore sizes > 10 nm showed 100% release in the first 2 days; however, when the pore size of PSi was reduced to < 6 nm, sustained release for up to two weeks was achieved.

In addition to the physicochemical properties of the proteins and peptides and the pore size of PSi, another important factor to be considered is the surface chemistry of PSi. Kovalainen *et al.* investigated the influence of the surface chemistry of PSi microparticles on the release of the peptide YY3-36 (PYY) [22]. Three different surface chemistries were studied, namely, (i) thermally oxidized PSi (TOPSi), (ii) thermally hydrocarbonized PSi

(THCPSi), and (iii) undecylenic acid treated THCPSi (UnTHCPSi). TOPSi presented a hydrophilic surface and THCPSi presented a hydrophobic surface, whereas UnTHCPSi resulted in a slightly more hydrophilic surface than THCPSi. The order of PYY release observed was TOPSi > THCPSi > UnTHCPSi. The rapid release from TOPSi was attributed to the hydrophilic nature of TOPSi, which allowed easy wettability and desorption of the peptide from the surface of the particles. However, in the case of THCPSi and UnTHCPSi, hydrophobic interactions between the peptide and pore surface, along with poor wettability, were possibly responsible for the delayed PYY release behavior. Moreover, the possible interaction of the carboxylic groups in UnTHCPSi with the amine groups of the peptide further sustained the PYY release [22]. Similarly, Jarvis *et al*. have also shown that by reducing the hydrophobicity of the surface of PSi by thermal oxidation, the hydrophobic interactions between the adsorbed protein and the PSi surface are lowered and the drug adsorption is mainly based on electrostatic interactions [200]. Thus, the aforementioned findings indicate that the surface chemistry of the PSi can be altered to achieve optimal loading and release profile for a given protein/peptide.

The composition and pH of the loading medium also have an influence on the loading of proteins and peptides. For example, the loading degree of human serum albumin was increased by up to 30% when the protein concentration was increased from 0.001 to 10 mg/mL [202]. Another important factor is the pH of the solution and the isoelectric point (pI) of the loading protein/peptide. When the pH of the loading medium is altered, the charge of the protein/peptide is also changed, which can influence the interaction between the protein/peptide and the PSi pore surface. Kaasalainen *et al*. studied the adsorption of GLP-1 into PSi NPs [199]. The study demonstrated that for hydrophobic particles, the highest loading degree was obtained at pH near the isoelectric point (pI) of the peptide. The study also showed that in the presence of strong interactions between the peptides and the PSi's surface, irreversibly and reversibly adsorbed drug layer was formed, as shown in the schematic representation in **Figure 7.** The study also highlighted the importance of peptide–peptide interactions in the pores, as the irreversibly adsorbed layer of the peptide forms a protective layer that facilitates the easy desorption of the reversibly adsorbed peptides [199].

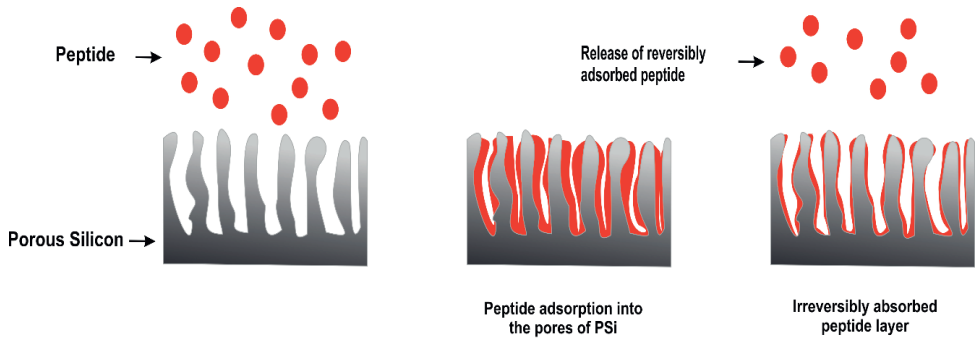


Figure 7 *Schematic representation of peptide loading into PSi, showing irreversible and reversible peptide adsorption into the pores of PSi.*

Based on the extensive studies on the loading of proteins and peptides into the PSi particles, PSi has emerged as a suitable protein and peptide delivery system. Furthermore, recent studies with advanced protein/peptide drug delivery systems based on PSi have also been reported. For example, Minardi *et al.* developed a composite microsphere comprising of poly(lactic-co-glycolic acid) (PLGA) as the outer shell and nanostructured silicon multistage vector as the core, for temporospatially controlled delivery of growth factors [24]. After subcutaneous injection to mice, the advanced system was able to retain the incorporated protein for more than 2 weeks, avoiding burst release. Likewise, Araujo *et al*. also developed a multifunctional composite system comprised of CPP and chitosan-modified PSi or PLGA NPs incorporated in stimuli-responsive polymeric microspheres [18]. The microsystem was assembled using the microfluidics technique and was aimed at orally delivering GLP-1. In the study, an enhanced *in vitro* GLP-1 intestinal permeability was observed along with high interactions with the intestinal cell lines.

2.9 Colloidal nanoparticles for oral protein and peptide delivery

The use of colloidal carriers, based on either polymeric or lipidic materials, have received a lot of attention because of their well accepted safety profiles [121]. These nanoparticulate systems offer several advantages as drug delivery systems, such as the ability to control their physicochemical properties (*e.g.*, size and charge), surface properties, release kinetics and site-specific action [102]. Emulsion polymerization, interfacial polymerization, emulsification evaporation, solvent displacement, salting out, emulsification diffusion, and desolvation are some of the examples of the preparation techniques used for these NPs [102, 205]. The selection of the preparation technique depends on the nature of the polymer or lipid used and the drug molecule. Nonetheless, most of these techniques involve heat, sonication, strong agitation or organic solvents, which can harm the structure of the encapsulated protein/peptide [88].

2.9.1 Polymeric nanoparticles

With the increase in understanding of the polymer structure and its properties, the diversity in the number of polymers available have notably escalated. This, in turn, has also helped in the development of various polymer-based NPs. Polymeric NPs are mainly classified as nanocapsules (vesicular structure with polymeric shell) and nanospheres (polymeric matrix) [12, 121, 206]. The ability to control the physicochemical properties of polymeric NPs to protect the encapsulated protein and peptide, to modulate their release behavior and to enhance their intestinal absorption, makes the polymeric NPs attractive carriers for proteins and peptides [88]. Some of the commonly used polymers for oral delivery are chitosan [207, 208], dextran [209], alginate [210], poly(γ-glutamic acid) (PGA) [211], hyaluronic acid [212], poly(lactic acid), PLGA [213], polycaprolactone [214], acrylic polymers [215], and polyallylamine [88, 216, 217]. With several beneficial properties, the polymeric NPs have also attracted attention from the pharmaceutical industry with several products under

preclinical and clinical studies. Some of the developed products are chitosan and PGA based polymeric nanospheres by NanoMega Medical Corporation (Lake Forest, CA, USA) [218], insulin-loaded nanospheres composed of patented biodegradable polymer by Aphios Corp. (Woburn, MA, USA) [219], and insulin-loaded bioadhesive NPs by NOD Pharmaceuticals, Inc. (San Diego, CA, USA) [220].

PLGA, an aliphatic polyester co-polymer, is one of the most used synthetic polymer for the oral delivery of insulin [100, 221]. The biocompatible and biodegradable nature of PLGA along with controlled drug release behavior has led to its widespread application as an oral protein and peptide delivery system, with several *in vitro* and *in vivo* studies demonstrating its efficiency [222-226]. The most common method to produce PLGA particles to encapsulate hydrophilic molecules is by the water-in-oil-in-water (w/o/w) double emulsion method [225]. However, poor drug loading (~1%) and possible drug degradation due to exposure to organic solvents and high shear stress during the preparation process are disadvantages associated with this technique.

In a study by Reix *et al*., PLGA NPs were able to withstand the conditions in the intestine allowing uptake and absorption of insulin [223]. After duodenal administration, the diabetic rats showed hypoglycemic effect similar to long acting insulin [223]. To impart the pH sensitivity to the PLGA NPs, several approaches have been studied, such as the use of the co-polymer of PLGA and Pluronic F68® [224], encapsulation of PLGA NPs into pH-sensitive polymers, such as the use of hydroxypropyl methylcellulose phthalate (HPMCP) and Eudragit RS [222]. The pH-responsive polymer coated NPs showed significant reduction in the burst release of insulin in gastric conditions, along with prolonged reduction in the blood glucose levels after oral administration [222]. The interaction of the PLGA with intestine can be improved by modification of its surface by using different moieties, such as chitosan, CPPs and targeting moieties. Recently, a multifunctional composite was developed with PLGA NPs that were modified using chitosan and CPP, and further coated with an enteric polymer, hydroxypropyl methylcellulose acetate succinate (HPMCAS) MF [18, 213, 227]. The NPs were able to release the loaded peptide GLP-1 only in intestinal conditions. Additionally, the CPP and chitosan modified PLGA NPs were able to show 5.6-fold higher interaction with the intestinal cell lines, Caco-2 and HT29-MTX, as compared to the unmodified NPs. Nonetheless, the efficiency of the system has yet to be proven in *in vivo* studies. Despite the large number of *in vitro* and *in vivo* studies, PLGA NPs have not been able to enter the clinical trials for oral protein and peptide delivery.

2.9.2 Lipid-based nanoparticles

Lipid-based carriers include a wide variety of systems, such as simple emulsions, self-emulsifying systems, liposomes, solid lipid NPs (SLNs), and nanostructured lipid carriers [136]. Such lipidic carriers have well-known safety profile, with some of them already established for large-scale production [228]. As compared to other systems, the SLNs have additional benefits to protect the encapsulated drugs, high stability and controlled drug release [229]. SLNs are solid lipids stabilized in aqueous solution in the presence of one or more surfactants. The proteins and peptides loaded in SLNs are mainly prepared by microemulsion, high-pressure homogenization, solvent evaporation and supercritical fluid

techniques [230]. The encapsulation and release of proteins and peptides from the SLNs are governed by several factors, such as the properties of the drug, lipid-matrix composition, type of surfactant size of NPs, preparation technique and its parameters. However, the SLNs are often associated with poor drug encapsulation and drug expulsion, which occur due to the polymorphic transition of lipids during the storage [229, 231].

SLNs composed of cetyl palmitate prepared by w/o/w double emulsion technique have been used for oral delivery of insulin. In the study, after oral administration, a biphasic hypoglycemic effect was observed, which was attributed to the biphasic release of insulin from the SLNs [232]. Another study compared the SLNs and wheat germ agglutinin modified SLNs containing insulin, both *in vitro* and *in vivo*. The study showed that the lectin-modified SLNs had improved stability and showed higher hypoglycemic effect as compared to the unmodified SLNs. However, poor bioavailability even after modification was observed, indicating the need for further development of the system [233]. Recently, a study reported stearic acid and octa-arginine CPP-modified SLNs for oral delivery of insulin [234]. Although the SLNs showed only partial protection of the encapsulated insulin against the enzymes in *in vitro* conditions, a significant hypoglycemic effect on diabetic rats was observed after oral administration. Nonetheless, the efficiency of these systems as oral protein/peptide carriers is yet to be proven in preclinical and clinical studies [234].

2.10 Biofunctionalization of the carrier systems

The first generation of drug delivery systems has shown its clinical applicability by enhancing the efficacy of several drugs [235]. However, to further improve the therapeutic efficacy of these drug delivery systems, researchers have explored a wide variety of surface biofunctionalization techniques and developed multifunctional multistage platforms for sustained release, targeting, and stimuli responsiveness [156, 195, 235, 236]. In the case of oral peptide delivery, these functionalities mainly include imparting mucoadhesivity to the carrier [237], targeting to intestinal cells [238], enhancing cellular interactions [239], permeation enhancing effect [17], and enteric coating [2, 131, 138, 240].

The following subsections deal with some of the modifications that can be used to enhance the performance of micro- and nanoparticles as oral protein/peptide delivery platforms.

2.10.1 Chitosan

Chitosan is a naturally occurring polysaccharide, β-(1→4) glucosamine polymer, found in a certain type of fungi [241]. However, it is mainly derived from deacetylation of chitin, which is the second most abundant polysaccharide in nature. The structure of chitin and chitosan are shown in **Figure 8** [241, 242].

The deacetylation, usually more than 50% of the entire polymer, is done at varying degrees and with different molecular weights, which have been known to influence the physicochemical properties, activity and degradation of chitosan. For example, the low

molecular weight chitosan with low degree of deacetylation has higher solubility and rate of degradation [243].

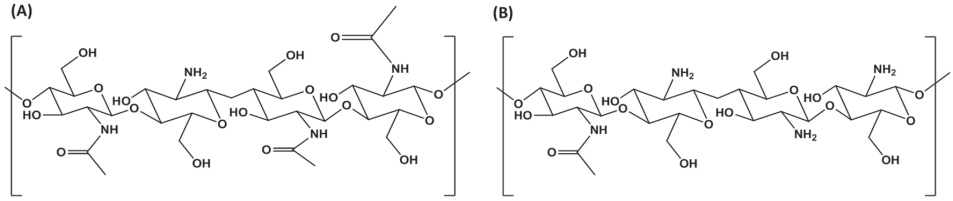


Figure 8 *Structure of (A) chitin and (B) chitosan.*

Chitosan is a cationic biopolymer with high positive charge density with each glucosamine residue containing one primary amine. The presence of such large number of amine groups confers important functional properties to chitosan, such as mucoadhesivity and permeation enhancement [242]. Chitosan is degraded in human tissues by lysozymes, with possible involvement of chitinases and acid hydrolysis in the process [244]. Owing to the biocompatibility and biodegradability, chitosan has been widely considered for drug delivery applications in the pharmaceutical field, but it has not been approved for this purpose yet [29]. Instead, chitosan has been approved for dietary applications in Japan, Italy and Finland [245], and it has also been approved by the FDA for wound dressing purposes [246]. Arai *et al*. reported that the lethal dose 50% (LD50) of oral chitosan was >16 g/kg, which is comparable to sucrose [247]. Furthermore, Sonaje *et al*. demonstrated that after oral administration of 100 mg/kg of chitosan-based NPs (80 kDa, 80% degree of deacetylation) to mice, no signs of oral toxicity were observed [248]. Nevertheless, the toxicity profile of chitosan can be altered by structural modifications, as despite the safety of the precursor, modification yields a completely new entity [244].

The mucoadhesive property of chitosan is mainly due to its electrostatic interactions with mucin, along with hydrophobic interactions and hydrogen bonding in the adhesion process [249]. The permeation enhancing effect of chitosan was established after the first reports about its ability to enhance the transmucosal delivery of proteins and peptides across the nasal epithelia [250], and enhancing the paracellular permeability of ^{14}C-mannitol across Caco-2 intestinal monolayers [251]. Since then, several *in vitro* and *in vivo* studies have been conducted to establish the permeation enhancing effect of chitosan [8]. The mechanism of chitosan's influence on the reversible TJs opening has been evaluated by several studies. In one of the studies in Caco-2 cell monolayers, chitosan treatment led to a decrease of the strength of TJ by redistribution of the transmembrane protein CLDN4 from the cell membrane to the cytosol, leading to its degradation in the lysosomes [252]. The TJs were recovered later by CLDN4 synthesis. However, the disruption of TJs seems to be a result of a cascade of reactions as elucidated by Hsu *et al*. (**Figure 9**) [253]. Briefly, the electrostatic interactions between chitosan and integrin present on the cell border lead to clustering, phosphorylation of focal adhesion kinases and Src tyrosine kinases. This, in turn, leads to the TJs disruption by redistribution of the CLDN4 transmembrane protein [253]. This permeation enhancing effect of chitosan is pH-dependent due to the low solubility of

chitosan at basic pH (pK_a ~6.6) [242]. With the increase in pH, chitosan loses its cationic nature, and thus, fails to interact with the integrin protein on the cell's surface, thereby preventing disruption of the TJs [254]. Conversely, another study reported the influence of the change in intracellular pH by activation of chloride-bicarbonate exchanger on the opening of the TJs by chitosan, without any changes in the TJs proteins (CLDN4 and ZO1) [255].

Owing to its beneficial properties, chitosan based drug delivery systems have been studied extensively for the oral delivery of proteins and peptides. Besides the use of chitosan micro- and nanoparticles for oral delivery of proteins and peptides [126, 240, 248, 256], chitosan polymer has also been used to modify the surface of other NPs to impart the mucoadhesive and permeation enhancer effects [18, 19, 257]. The use of chitosan to modify the surface of other NPs would combine the benefits of two systems. For example, Fonte *et al.* prepared insulin–loaded SLNs with negatively charged surface, onto which chitosan was adsorbed, conferring positive charge to the NPs. The chitosan coated SLNs showed higher insulin permeability across intestinal cell monolayers and showed more pronounced hypoglycemic effect in diabetic rats as compared to unmodified SLNs [19]. Similarly, chitosan was used to coat nanoemulsions and lipid NPs, which showed improved oral absorption of the encapsulated protein and peptide [19, 258]. However, despite the advantages associated with chitosan-based systems, it is still susceptible for degradation after exposure to the harsh acidic condition in the stomach.

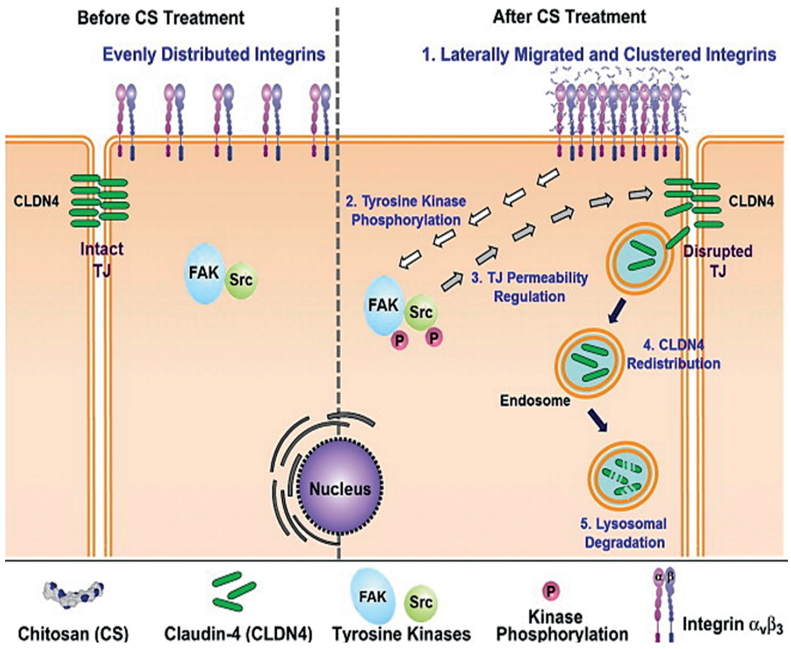


Figure 9 *Schematic representation of signaling mechanism of chitosan-mediated TJs opening [253].*

2.10.2 Thiolated chitosan

Thiolation of chitosan denotes the modification of the primary amine groups with thiol-containing molecules, such as cysteine, thioglycolic acid, and 2-iminothiolane [259]. There are several advantages of thiolated chitosan as compared to the unmodified forms, such as, enhanced mucoadhesivity due to the formation of disulfide bonds with the mucus glycoproteins, and *in situ* gelling because of the pH-dependent inter- and intramolecular disulfide bonds formation [30, 260] In addition, thiomers have also been shown to have a permeation enhancing effect. The suggested mechanism for permeation enhancing effect is based on the formation of the disulfide bond between the cysteine residues on thiomers and the reduced glutathione presence on the apical mucosa of the intestine [30, 261, 262]. The reduced glutathione inhibits the enzyme, protein tyrosine phosphatase, which subsequently leads to phosphorylation of the tyrosine subunits of occludens. This phosphorylation leads to the opening of the tight junctions, thus, exhibiting the permeation enhancing effect [30]. Moreover, thiolated chitosan has also been reported to inhibit the ATPase activity of p-glycoprotein in intestine [263, 264].

Because of the abovementioned beneficial properties, thiolated chitosan have been widely used for the oral delivery of proteins and peptides [265-267]. Recently, thiolated chitosan-coated liposomes have been shown as an efficient peptide carrier, augmenting the oral bioavailability of the loaded peptide drug [268]. Another study by Millotti *et al*. also showed that after oral administration of chitosan-6-mercaptonicotinic acid NPs, the area under the curve of the plasma insulin levels were 4-fold higher as compared to unmodified chitosan NPs [239].

2.10.3 Cell-penetrating peptides (CPPs)

CPPs, also known as Trojan horse peptides, have also attracted a lot of interest, owing to their ability to enter inside the cells. CPPs, 5–30 amino acid peptide sequences, are known for their ability to deliver a wide variety of cargos into the cells, such as small molecules, proteins and peptides, nucleic acids and NPs [269]. Since the first report of use of the human immunodeficiency virus TAT (Trans-activator of transcription) protein to enhance the absorption of a protein molecule after an intraperitoneal injection [270], the use of CPPs has received considerable attention as permeation enhancers for proteins and peptides. Some of the examples of CPPs exploited for oral delivery of proteins and peptides, besides TAT, are oligoarginine and penetratin [271]. These CPPs are reported to enhance the intestinal permeation induced by adsorption to the cell surface glycosaminoglycans [271].

One approach for oral delivery of drugs includes the co-administration of the CPP with proteins and peptides, either by forming a chemical conjugation between them [272] or by using a physical mixture [273]. Another approach includes the modification of the surface on the nanocarriers loaded with proteins and peptides [20]. The latter approach with modification of the nanosystem can be advantageous as it avoids any alteration to the structure of the protein and peptide. Liu *et al*. developed oligoarginine-modified polyethylene glycol (PEG) PLGA NPs for oral insulin delivery. The nanosystem improved the hypoglycemic effect and augmented the relative oral bioavailability of insulin, without

any signs of histological toxicities in the ileac mucosal membranes [20]. Similarly, enhanced cellular internalization effect of CPP-modified PLGA NPs have also been described [274]. CPP has also been used to modify chitosan-based NPs with the aim to combine both of their effects [18, 275, 276]. Guo *et al*. showed that TAT and chitosan derived poly(vinyl alcohol) (PVA) NPs enhanced cellular uptake and transcellular transport of insulin [275]. Moreover, the TAT- and chitosan-modified PVA NPs were able to display enhanced hypoglycemic effect in diabetic rats as compared to only chitosan-modified PVA NPs and unmodified PVA NPs. In addition, the colon specific targeting of the system was shown by single-photon emission computer tomography of the ^{99m}Tc isotope labelled NPs after oral gavage in minipigs [275].

2.10.4 Enteric coating of nanoparticles

Apart from the modification of the particulate systems to enhance the intestinal permeability, it is also necessary to protect the encapsulated proteins/peptides against the harsh conditions of the gastric milieu [10]. Taking advantage of the high pH variations in the GI tract, pH-responsive systems can be developed to avoid the release of proteins/peptides in stomach with site-specific release in the small or large intestine. Such pH-responsive systems can be prepared either by coating the NPs with pH sensitive polymer or by incorporating such polymers in the system to induce pH responsivity [10, 17, 128].

There are several FDA-approved commercially available pH-responsive polymers, such as poly(methacrylic acid-*co*-methyl methacrylate) (Eudragit L, S and F), HPMCP and HPMCAS [277]. These polymers are insoluble at low pH but are soluble at higher pH. In the case of HPMCAS, the polymer dissolving pH can be adjusted by altering the amounts of methoxyl, hydroxypropyl, acetyl and succinoyl groups on the cellulose backbone of polymer [278]. HPMCAS was originally developed and marketed by Shin-Etsu Chemical Co., Ltd. (Japan) in 1986 [279]. It is categorized based on the physicochemical properties in six grades. The F grade represents fine particles and the G grade represents the granular particles. Additionally, the L, M and H grades are chemically different exhibiting different solubility at various pH, where L, M and H dissolves at pH $\geq$ 5.5, 6.0, and 6.8, respectively [278, 279]. HPMCAS polymers have been used in several studies to develop pH responsive systems [280-282].

A number of techniques have been investigated for coating NPs, such as the layer-by-layer technology [283], physical adsorption [284], supercritical anti-solvent process [285], and microfluidics [286]. However, this dissertation focuses on a new approach to coat NPs, known as AFR technology. AFR technology is a simple, single-step method [33]. A schematic representation of the AFR system is shown in **Figure 10**. The AFR reactor consists of a feeding container, an atomizer, a heating tube, a diluter and a sample collector. The first step involves the input of an aqueous or non-aqueous feed dispersion comprising of the drug and polymer, or the NPs and polymer, into the feeding chamber. Then, the fed dispersion is atomized into small droplets in the atomizer, which are then carried to a heated tubular reactor by the stream of nitrogen gas. Thus, the formed droplets are dried in an upward stream of nitrogen gas, whilst passing through the heated tube. The temperature and residence time of the droplets can be easily controlled, thus allowing close control of the

physicochemical properties of the produced NPs [38, 287]. AFR technology has been employed to prepare different types of micro- and nanosystems for drug delivery purposes. Recently, Sapra *et al*. developed spherical lipid NPs with *in situ* surface modification using the AFR technology [288]. The surface modified NPs showed enhanced suspension stability for up to 30 days, by using minimal amount of surface-active agents [288]. In another study by Raula *et al*., stable microparticles were developed for pulmonary delivery of two drugs with different water solubilities [35]. The intact microparticle was composed of a nanosuspension of budesonide and salbutamol sulfate with mannitol as bulking agent, and leucine as coating agent. Thus, the formed microparticles allowed simultaneous delivery of two drugs and displayed excellent aerosolization properties, exhibiting potential for inhalation therapy [35]. Moreover, this technology has also been employed to generate solid state DNA NPs (65–125 nm). The DNA content in these NPs was ~10% and was shown to be stable at room temperature [34].

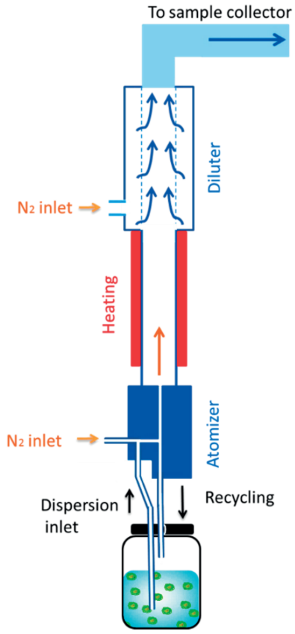


Figure 10 *A schematic illustration of the AFR technology.*

AFR technology also has additional benefits for preparing formulations for oral peptide and protein delivery, such as the possibility to avoid the use of any organic solvents by preparing the dispersion feed in an aqueous medium. In addition, despite of heat application, the residence time of the droplets in the heated tube lasts only tens of seconds, thus, avoiding any heat-associated damage [287]. The simplicity and control afforded by the AFR technology makes it a potential candidate as a polymeric coating approach for protein/peptide encapsulated NPs.

In summary, the abovementioned different strategies and modification approaches can be utilized to develop potential carriers for oral delivery of proteins and peptides.

3 Aims of the study

Despite decades of time and efforts invested, the development oral protein/peptide delivery systems is still a major challenge. PSi has been studied extensively for its ability to efficiently load and deliver different types of cargos, from small molecules to large biomacromolecules. Hence, taking advantage of the numerous benefits associated with the PSi system, this dissertation aimed at developing several PSi-based carriers for oral delivery of insulin and GLP-1, which would be able to overcome the limitations associated with the oral protein/peptide delivery for the treatment of DM. Several surface modification approaches to impart mucoadhesivity, permeation enhancement and pH-sensitivity to the PSi systems were also developed and assessed.

The specific objectives of this dissertation were:

1. To investigate the influence of different surface chemistries of PSi microparticles and different chitosan modification techniques on the interactions with intestinal cells and insulin permeability across intestinal Caco-2 and HT29 cell monolayers. (**I**)

2. To study and compare several chitosan coated nanocarriers based on polymer, lipid and PSi on the possibility to load GLP-1 and transport it across the intestinal Caco-2/HT29-MTX cell monolayers. (**II**)

3. To fabricate multistage mucoadhesive polymer/PSi nanocarriers using AFR technology for dual-drug loading and site-specific intestinal delivery, enhancement of the cellular interactions and augmented intestinal GLP-1 permeation across Caco-2/HT29-MTX/Raji B cell monolayers. (**III**)

4. To assess *ex vivo* the ability of multistage pH-responsive polymers/PSi nanocarriers loaded with GLP-1 and DPP4 inhibitor to enhance the permeation of GLP-1 across rat intestinal tissues, as well as to study the nanocarriers' *in vivo* behavior in diabetic rats. (**IV**)

5. To potentiate the permeation enhancing effect of chitosan-modified PSi NPs by further surface modifications with L-cysteine and CPP, and, to study the insulin uptake and transport mechanism across intestinal Caco-2/HT29-MTX/Raji B cell monolayers. (**V**)

4 Experimental

This section summarizes the experimental methods used in this dissertation. A detailed description of all the materials, equipment and methods used in this work can be found in the respective original publications (**I–V**). The primary surface stabilized PSi micro- and nanoparticles were produced at the Laboratory of Industrial Physics, Department of Physics and Astronomy, University of Turku, Finland.

4.1 Preparation of micro- and nanoparticles

4.1.1 Fabrication of PSi micro- and nanoparticles (I–V)

A monocrystalline, boron doped p+-type Si ⟨100⟩ wafers of 0.01–0.02 Ω cm resistivity were used to prepare the PSi layers using electrochemical anodization [25]. A schematic representation of the electrochemical anodization cell used in this work is shown in **Figure 6**. The electrolyte solution used consisted of 1:1 (v/v) hydrofluoric acid (HF, 38%)–ethanol solution. The fracture planes in the Si-wafers were created by subjecting the wafer to repeated and pulsed low/high etching profile. Then, the porous layer was lifted off from the Si substrate by sharply increasing the etching current to the electropolishing region. The surface of the PSi were then stabilized by thermal hydrocarbonization by placing the porous layer into a quartz tube in nitrogen flow (1 L/min) for at least 30 min to remove the residual moisture and oxygen. Then, an acetylene flow (1 L/min) was added to the nitrogen flow at room temperature, first for 15 min and then at 500 °C for the next 15 min, whilst maintaining 1:1 (v/v) nitrogen and acetylene flow. The layers were cooled down to room temperature under nitrogen flow. This process yielded thermally hydrocarbonized porous silicon (THCPSi) (**I**). For the undecylenic acid treatment, the THCPSi layers were immersed into undecylenic acid for 16 h at 120 °C, yielding an undecylenic acid modified THCPSi (**I–V**) surface. Publications **I–II** use UnTHCPSi as an abbreviation for undecylenic acid modified THCPSi and publications **III–V** use the abbreviation UnPSi.

To prepare the annealed PSi materials, the as-anodized PSi layers were placed into a quartz tube under nitrogen flush for 45 min at room temperature followed by placing the quartz tube containing PSi layers to a 700 °C furnace for 15 min [22, 289]. All the surface stabilization with thermal hydrocarbonization and undecylenic treatment were performed after this step to form annealed THCPSi (AnnTHCPSi) and annealed UnTHCPSi (AnnUnTHCPSi) (**I**).

Finally, to yield micro- and nanoparticles, the multilayer films were wet milled using high-energy ball mill (**I–V**). The particle size fraction collected for the microparticles was <25 μm after wet sieving with ethanol (**I**). The final NPs were obtained by using centrifugation (**II–V**)**.**

4.1.2 Fabrication of polymer- and lipid-based nanocarriers (II)

A modified solvent emulsification–evaporation method based on the w/o/w double emulsion technique was used to prepare polymeric and lipidic NPs [233]. PLGA 50:50 was obtained from Purac Biomaterials, Purasorb® PDLG 5004 and Witepsol E85 from Sasol, Germany. First, the polymer/lipid solution (100 mg/mL) was prepared in ethyl acetate in a heated water bath. To this solution, 100 µL of GLP-1 solution (2.5 mg/mL) was added and the primary water-in-oil (w/o) emulsion was prepared by homogenization for 30 s using Vibra-Cell™ ultrasonic processor (Sonics®, Sonics and Materials, Inc., USA). The primary emulsion was added to 4 mL of PVA solution (2 %), and again homogenized for 30 s to form the second w/o/w emulsion. This second emulsion was then added to 7.5 mL of 2% PVA solution and was left for 3 h under stirring to allow the organic solvent to evaporate. Empty NPs were prepared by replacement of GLP-1 solution with Milli-Q water and following the same procedure as mentioned above.

4.2 Surface modification of the particles

4.2.1 Chitosan modification of PSi microparticles (I)

AnnUnTHCPSi microparticles were modified with chitosan by either physical adsorption or chemical conjugation. For physical adsorption, 5 mg of insulin loaded microparticles were dispersed in 1 mL of chitosan solution (20 mg/mL in 0.01% acetic acid), and then stirred for 3 h at room temperature. After that, the microparticles were washed twice with 0.01% acetic acid and Milli-Q water and separated by centrifugation at 4500 × *g* for 3 min.

For chemical crosslinking of chitosan to the surface of AnnUnTHCPSi NPs, carbodiimide crosslinker with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were used. First, 3 mg of microparticles were dispersed in 8 mL of 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.2) containing EDC/NHS (molar ratio 1:1.12), and then sonicated to disperse the microparticles and stirred for 2 h in dark conditions. After 2 h of stirring, 2 mL chitosan solution (6 mg/mL in 0.01% acetic acid pH 5.2) was added to the activated microparticles and stirred overnight to allow conjugation. The microparticles were washed similarly to the physical adsorption testing.

After washing, both the NPs were dried overnight at room temperature and the dried samples were stored at 4 °C until further use.

4.2.2 Chitosan modification of polymeric, lipidic and PSi nanoparticles (II)

Chitosan was physically adsorbed onto the polymeric, lipidic and PSi NPs to modify their surfaces. For this, 1 mg of NPs were dispersed in 0.5 mL of 1% chitosan solution (in 1% acetic acid at pH 5.5) and were allowed to stir overnight at room temperature for deposition of chitosan on the surface of the particles. The NPs were washed three times using Milli-Q

water and separated using centrifugation (Optima™ TL, Beckman Coulter, USA). After washing, the NPs were re-dispersed in Milli-Q water and stored at 4 °C until further use.

4.2.3 Chitosan, L-cysteine and CPP modification of PSi nanoparticles (III–V)

Chitosan was covalently linked to the UnPSi NPs (**III–V**) using EDC/NHS crosslinking to form an amide bond between UnPSi and chitosan, forming chitosan-modified UnPSi (CSUn). For this, 1.5 mg of UnPSi NPs were dispersed in 4 mL of 10 mM MES buffer, and evenly dispersed by sonicating for 30 s. Then, 8 µL of EDC (0.877 g/mL at 20 °C) and 6 mg of NHS were added to the dispersion and stirred for 2 h at room temperature in dark to activate the carboxylic groups of the UnPSi NPs. After activation, the NPs were again tip-sonicated for 30 s to disperse the NPs properly. To the dispersion, 0.6 mL of 5 mg/mL chitosan solution (in 1% acetic acid at pH 5.2) were added and stirred overnight to allow an efficient conjugation to the particles. The NPs were washed three times with Milli-Q water with subsequent centrifugation to remove the supernatant containing excess of chitosan. The CSUn NPs were dispersed in Milli-Q water and stored at 4 °C until further use.

L-cysteine and CPP modifications of CSUn NPs were also performed by EDC/NHS chemistry. 1.5 mg of CSUn NPs were dispersed in 4 mL of 10 mM of MES buffer solution with 8 µL of EDC and 6 mg of NHS with a final pH of 5.2, and tip sonicated for 30 s. Then, 15 mg of cysteine or 0.5 mg of CPP was added to the dispersion to form cysteine modified PSi (CYS-CSUn) or CPP modified PSi (CPP-CSUn). The NPs were allowed to mix overnight at room temperature in dark conditions, and then washed twice with Milli-Q water with subsequent centrifugation to remove unbound L-cysteine or CPP. The modified NPs were dispersed in Milli-Q water and stored at 4 °C until further use.

4.2.4 Alexa Fluor 488 modification of PSi nanoparticles (III–V)

Alexa Fluor 488 (AF488) conjugated NPs were used for cellular interaction studies, which are explained in detail in later sections. First, AF488-conjugated UnPSi NPs were prepared by surface activation as described in *Section 4.2.3*. After activation, 21 µg of AF488 was added to NPs and stirred for 2 h. The NPs were washed once with diluted hydrochloric acid (0.01 M) and once with water. Then, the AF488-conjugated UnPSi were treated as UnPSi NPs and further treated with chitosan, L-cysteine and CPP, as described above, to obtain the corresponding AF488 modified NPs.

4.2.5 Coating by AFR technology (III–IV)

A dispersion consisting of 3 mg of GLP-1 loaded CSUn NPs, 15 mg of HPMCAS-MF polymer and 2 mg of DPP4 inhibitor solution was prepared, with the total solid content of 0.01% (w/v) at pH 7.4. The NPs dispersion was sonicated for 1 min, and then fed into the AFR. A collision-type jet atomizer breaks the feed into droplets, which are then suspended using nitrogen gas (3 L/min), which carries the droplets into the heated laminar flow reactor

(80 °C). In the reactor, the droplets were dried to complete the formation of HPMCAS-MF coated CSUn (H-CSUn) [37, 287], which were then collected in a Berner-type low-pressure impactor [290]. A schematic representation of the AFR technology and the process involved is shown in **Figure 9**.

4.3 Physicochemical characterization

The physical properties of the PSi micro- and nanoparticles, such as the pore size and volume, and the surface area were characterized by nitrogen sorption using a TriStar 3000 gas sorption apparatus. The specific surface area was calculated using the Brunauer-Emmett-Teller theory from the acquired isotherms and the pore volume was calculated using the total adsorbed amount at a relative pressure of $p/p_0 = 0.97$ [291]. Additionally, the average pore diameter was also estimated using the surface area and the total pore volume, assuming that the pores were cylindrical in shape [21].

The size (*z*-average) distribution for the NPs and zeta-potential (ζ-potential) for both microparticles and NPs were measured using a Zetasizer Nano instrument (Malvern Ltd., UK). The surface modification of the prepared systems were confirmed by analyzing dried micro- and nanoparticles using Fourier transform infrared spectroscopy. The morphology of the microparticles was investigated using a scanning electron microscope (SEM, Zeiss DSM 962) after sputter coating with Pt. The NPs were also visualized under transmission electron microscope (TEM, Tecnai™ F12, FEI Company, USA). The multistage pH-polymer NPs were evaluated by high-resolution scanning electron microscope (HR-SEM, Zeiss Sigma, Germany). The H-CSUn NPs were placed on stubs and then sputter coated with gold before observation under HR-SEM.

4.4 Drug loading and release

4.4.1 Insulin loading and release from PSi microparticles (I) and nanoparticles (V)

Drug loadings of the micro- and nanoparticles were performed using an immersion technique [25]. For microparticles, 1.25 mg of microparticles were dispersed in an insulin solution (5 mg/mL in 0.01 M of HCl) and were mixed for 90 min at room temperature. The microparticles were then centrifuged to remove the excess of unloaded insulin. The supernatant was used to quantify the amount of unloaded insulin by high performance liquid chromatography (HPLC) (**I**). For the chitosan-adsorbed particles, the loading was performed before the surface modification, whereas for the chitosan-conjugated particles, the loading was performed at the end following the same protocol. The drug loading was calculated using **Eq. (1)**:

Insulin was loaded into the modified NPs by first dispersing 200 µL of NPs (2 mg/mL) into 900 µL of insulin solution (200 µg/mL), followed by stirring at room temperature for

90 min. The NPs were centrifuged to remove the excess of insulin and washed once with Milli-Q water. The drug loading was calculated using **Eq. (1)**.

$$\text{Loading degree (\%)} = \frac{\text{Initial amount of drug added } (\mu g) - \text{Free unloaded drug } (\mu g)}{\text{Weight the particles } (\mu g)} \times 100\%$$

Eq. (1)

The *in vitro* release profiles of insulin from the modified NPs were investigated by dispersing the NPs equivalent to 35 µg of insulin in 10 mL simulated gastric fluid (SGF, without pepsin) for the first 2 h. Then, the NPs were removed from the medium by centrifugation and reintroduced in 10 mL of fasted state simulated intestinal fluid (FaSSIF) for the next 6 h. Samples were withdrawn at different time intervals, and the volume was replaced with fresh pre-warmed medium after each withdrawal. HPLC was used to quantify the amount of insulin released over time (**V**).

4.4.2 GLP-1 loading and release studies from polymeric, lipidic and PSi nanoparticles (II)

In the case of polymeric and lipidic NPs, GLP-1 was loaded during the particle's preparation step as described in *Section 4.1.2*. For PSi NPs, 1 mg of the NPs was dispersed in solution containing 200 µg of GLP-1. The dispersion were stirred for 90 min at room temperature, and then centrifuged after that to remove the excess amount of GLP-1. Chitosan coating was performed onto PSi NPs after the loading process. GLP-1 loading was evaluated by quantifying the amount of GLP-1 in the supernatant for all the three NPs (**II**).

For the release study, the amount of NPs corresponding to 30 µg of GLP-1 was first dispersed in pH 1.2 buffer for 2 h and then was transferred to FaSSIF for the next 4 h, to simulate the passage of the NPs in the GI tract. The experiment was performed at 37 °C and the samples were withdrawn at different time points followed by replacement with fresh pre-warmed release media. The samples were centrifuged and then the supernatants were analyzed in HPLC to quantify the amount of GLP-1 released (**II**).

4.4.3 GLP-1 and DPP4 inhibitor loading and release from polymer-coated PSi nanoparticles (III–IV)

GLP-1 was loaded into the pores of UnPSi and CSUn NPs, and DPP4 inhibitor was encapsulated in the polymeric matrix coating (**III and IV**). 1 mg of NPs were dispersed in 1.5 mL of GLP-1 solutions (133 µg/mL), which were magnetically stirred for 90 min at room temperature. After loading, the NPs were centrifuged to remove the excess of GLP-1. The loading degree for GLP-1 was calculated using **Eq. (1)**. For DPP4 inhibitor, 1 mL of the inhibitor solution (1 mg/mL in Milli-Q water) was added to 6 mL of HPMCAS-MF

solution. The final polymer coated NPs was dissolved in phosphate buffer (pH 7.4) to determine the loading degree of DPP4 inhibitor.

The *in vitro* release behaviors of GLP-1 and DPP4 inhibitor were studied by dispersing the NPs in two different release media: 10 mL of pH 1.2-buffer solution for 2 h and 10 mL of FaSSIF (pH 6.8) for 6 h at 37 °C. For the GLP-1 release studies, the amount of NPs equivalent to 30 µg of GLP-1 was used, and for the DPP4 inhibitor release study, NPs corresponding to 30 µg of DPP4 inhibitor were used. The sample aliquots were collected at different time points, and were replaced by fresh pre-warmed buffer. The samples were immediately centrifuged, and the aliquots were analyzed by HPLC (**III**).

4.5 *In vitro* cell based studies

4.5.1 Cell lines and cell culturing (I–III, and V)

Human colon adenocarcinoma Caco-2, goblet cells HT29, lymphocytes Raji B, and human gastric adenocarcinoma AGS cells were obtained from American Type Culture Collection (USA). Goblet-like HT29-MTX was kindly provided by Dr. T. Lesuffleur (INSERM U178, Villejuif, France). All the used cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM), except the AGS cells that were cultured in Roswell Park Memorial Institute (RPMI) medium. All the cell cultures were kept in a standard incubator (16 BB gas, Heraeus Instruments GmbH, Germany) at 37 °C in an atmosphere of 5 % CO_2 and 95 % relative humidity. Both media were supplemented with 10% heat inactivated fetal bovine serum, 1% non-essential amino acid, 1% L-glutamine, penicillin (100 IU/mL) and streptomycin (100 mg/ml). The growth medium was changed every other day for all the cell lines used until the end of the experiments, and the subculturing performed at 80% confluency. The adherent cells were passaged using trypsin-PBS-EDTA.

4.5.2 Cytotoxicity

For the cytotoxicity studies, cells were first seeded into 96-well plates (Corning Inc., USA). Caco-2 and HT29 cells were seeded at a cell density of 1×10^6 cells/mL (**I**), and AGS, Caco-2 and HT29-MTX were seeded at a cell density of 5×10^5 cells/mL (**II–V)**. The cells were allowed to attach for 24 h, and were washed with Hank's balanced salt solution (HBSS)–2-(4-(2-hydroxyethyl) piperazin-1-yl) ethanesulfonic acid (HEPES) buffer (pH 7.4) before adding the particles. 100 µL of the particle dispersions in HBSS–HEPES buffer were added to the wells and were incubated for 3 h for AGS cells (**III**) and 12 h for the intestinal cells (**I–V**) at 37 °C. After incubation, the cells were washed once with fresh HBSS–HEPES buffer. Next, 100 µL of CellTiter-Glo® reagent assay (Promega Corporation, USA) pre-diluted with HBSS–HEPES buffer (2×) were added to the wells. A positive (1% Triton X-100) and negative control (HBSS–HEPES buffer solution) were prepared and treated similarly to the sample wells. The luminescence was measured using Varioskan Flash

Multimode Reader (Thermo Fisher Scientific, USA). All the experiments were carried out at least in triplicate ($n \geq 3$).

4.5.3 Cell–particle interactions

The cell–particle interactions were evaluated qualitatively by confocal fluorescence microscopy and TEM imaging, and quantitatively by flow cytometry. Fluorescein isothiocyanate (FITC)-insulin loaded microparticles (**I**), FITC loaded NPs (**II**) or AF488-conjugated NPs (**III and V**) were used for the interaction studies with Leica SP5 confocal fluorescence microscopy (Leica Microsystems, Germany).

For confocal studies, intestinal cells either as co-cultures (**I–III**) in a ratio of 9:1 (Caco-2:HT29/HT29-MTX) or as monocultures (**III**) were seeded in Lab-Tek™ 8-chamber slides (Thermo Fisher Scientific, USA), and were allowed to attach for 24 h. The cells were then washed with pre-warmed buffer; 200 µL of particles (250 µg/mL) were added to the cells, and incubated for 3 h at 37 °C. After the end of incubation period, the cells were washed twice with fresh HBSS–HEPES buffer. The plasma membranes were stained by incubating the cells with 200 µL of CellMask™ Orange (Invitrogen™, USA) for 3 min at 37 °C. The excess of staining solution was washed twice with fresh HBSS–HEPES buffer and the cells were fixed by incubating with 2.5% glutaraldehyde for 20 min at room temperature. The intracellular localization of the particles was then observed by confocal fluorescence microscope. A triple co-culture cell monolayer composed of Caco-2/HT29-MTX/RajiB cells (90:10:1) was used to study the cell–particle interactions (**V**) [292, 293]. The NPs were incubated with the cell monolayers for 3 h at 37 °C. After incubation, the cell monolayers were washed with the fresh HBSS–HEPES buffer and the glycoproteins stained with wheat germ agglutinin (WGA) labelled with Alexa Fluor 594 (10 µg/mL) for 10 min at 37 °C. The cell monolayers were immediately observed under confocal fluorescence microscope, where *z*-stack images were taken and later analyzed with Imaris software (Bitplane, USA) to develop 3D images.

For the TEM imaging analysis, the particles were incubated with cell monolayers for 3 h at 37 °C, and then washed twice with HBSS–HEPES buffer. The cell monolayers were fixed with 2.5% glutaraldehyde for 20 min at room temperature, and washed twice with sodium cacodylate buffer for 3 min. Next, the cell monolayers were post-fixed with 1% osmium tetroxide in 0.1 M NaCac buffer (pH 7.4), and dehydrated and embedded in epoxy resin. Ultrathin sections (60 nm) were cut perpendicularly to inserts, post-stained with uranyl acetate and lead citrate, and examined under TEM (Tecnai™ F12, FEI Company, USA).

For quantitative analysis of the cell–particle interactions, flow cytometry studies (**III and V**) were conducted. The intestinal cells were first seeded in 6-well plates at a density of 7×10^5 cells/well and incubated at 37 °C for two overnights. The cells were washed once with HBSS–HEPES (pH 7.4) buffer. Next, 1.5 mL of particles of AF488-conjugated NPs were added to the cells and incubated for 3 h at 37 °C. After incubation, the cells were washed twice with the buffer and detached with trypsin-EDTA (**III**) or Versene (**V**). After detaching, the cells were once again washed with the buffer, suspended in 700 µL of buffer and measured with Gallios Flow Cytometer (Beckman Coulter, USA) with an excitation

laser wavelength of 488 nm. For each sample, ca. 10,000 events were recorded, analyzed, and plotted using Kaluza Analysis Software v1.3 (Beckman Coulter, USA).

4.5.4 Cellular intestinal permeability

For evaluating the intestinal permeability of insulin and GLP-1, two different cellular intestinal models were prepared. For the first study, Caco-2/HT29 (**I**) co-culture monolayers were prepared. 500 µL of cells (1.5×10^5 cells/mL) were seeded in 12-well cell culture inserts (Transwell®, Corning, USA), with the cell medium replaced every other day until the cell monolayer was formed after 21–22 days. For the other studies (**II, III and V**), Caco-2/HT29-MTX/RajiB triple co-culture cell monolayers were prepared. In this model, Caco-2 and HT29-MTX were seeded as the cell co-cultures described above, and maintained until 14 days. Then, RajiB cells (1×10^5 cells/well) were added to the basolateral chamber and the cell monolayers were maintained for the next 7 days. Raji B was added to induce M cell phenotype in Caco-2 cells in the monolayer [292]. During the permeability studies, the cell monolayers were first washed twice with HBSS–HEPES buffer and then 0.5 mL of the particle dispersion were added to the apical chamber. The plates were then incubated at 37 °C for 3 h with shaking at 100 rpm. Samples (200 µL) were withdrawn from the basolateral chamber at different time intervals, and then replaced with fresh pre-warmed buffer each time to maintain constant medium volume during the experiments. Insulin was quantified using a human insulin Enzyme-Linked Immunosorbent Assay (ELISA) kit (Mercodia, Sweden), GLP-1 was quantified using a GLP-1 Enzyme Immunoassay (EIA) kit (Sigma Aldrich, USA), and DPP4 inhibitor was quantified using HPLC. The activity of the DPP4 enzyme was analyzed using a DPP4 assay kit (Sigma–Aldrich, USA) to confirm the enzyme inhibition after the exposure to the DPP4 inhibitor.

4.5.5 Cellular intestinal uptake and transport mechanism (I and V)

The cellular uptake of FITC-insulin loaded PSi microparticles were studied with Caco-2/HT29 cell monolayers (**I**). PSi microparticles were added to the apical side of the monolayer and the HBSS–HEPES buffer was added to the basolateral chamber. The particles were then incubated with the cell monolayer at 37 °C for 3 h with shaking at 100 rpm. Then, the cells were first washed, lysed using a cell-lysing buffer, and then the lysate was centrifuged to remove the supernatant. The supernatant was used to quantify the amount of insulin interacting with the cells and the mucus layer by using a Varioskan Flash Multimode Reader.

The cellular uptake and transport mechanism studies were also performed in a triple co-culture intestinal cell monolayer model (as described above) for the L-cysteine- and CPP-modified CSUn NPs (**V**). Firstly, the cell monolayers were washed and equilibrated with the buffer for 15 min at 37 °C. Then, 0.5 mL of NPs (250 µg/mL) were added to the apical chamber under different conditions and also in the presence of several inhibitors: (i) 4 °C (ii) 100 mM of sodium azide (iii) 1 mM of protamine (iv) pre-incubation with 35 mM of sodium chlorate for 48 h and rinsed twice with HBSS–HEPES buffer and incubated with

the NPs dispersion (v) chlorpromazine (10 µg/mL) (vi) fillipin (1 µg/mL) and (vii) controls without any inhibitor. All the inhibitors were added with the NPs and incubated with the cell monolayers for 3 h at 37 °C [238]. After the incubation period, the amount of insulin in the basolateral chamber was quantified for all the tested conditions using a human insulin Elisa Kit (Mercodia, Sweden). The results are shown as percentages compared to controls (without any inhibition denotes 100%). The cell monolayers were then washed three times with ice-cold HBSS–HEPES buffer, including a wash with 5 mM of EDTA solution to remove the adhering NPs. The cells were lysed by incubating with the cell lysis buffer, and then the lysate was centrifuged at 4000×*g* for 5 min. The supernatants were analyzed to quantify insulin. The cell associate proteins were also analyzed in the lysate using a Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, USA). The uptake results are shown as the percentage of the control of insulin associated per mg of cell protein.

4.6 *Ex vivo* permeability and intestinal adhesion studies (IV)

Ex vivo permeability of GLP-1 and DPP4 inhibitor across healthy rat ileum were studied using a ligated intestinal method as described by *Yin et al* [126]. A freshly excised small intestine of rat was cleaned using Kreb's ringer buffer, and sections of ca. 5 cm were cut, with one end closed using a thread. Then, H-CSUn NPs containing 50 µg of GLP-1 and 180 µg of DPP4 inhibitor dispersed in 0.5 mL of Kreb's-Ringer buffer was placed into the intestinal loop. The loop was closed by tying the open end with silk thread to form a closed sac. As a control, 0.5 mL of solution containing GLP-1 (100 µg/mL) and DPP4 inhibitor (360 µg/mL) in pre-oxygenated Kreb's-Ringer buffer was used. The filled intestinal sacs were washed several times with pre-oxygenated Krebs-Ringer buffer, and then immersed in container with 10 mL of Kreb's ringer buffer at 37°C with smooth shaking. The buffer was aerated throughout the experiment with 95% oxygen and 5% carbon dioxide. Samples (200 µL) were withdrawn from serosal side at regular time intervals (up to 3 h) and the withdrawn volume was replaced subsequently with pre-warmed fresh buffer. The GLP-1 content was analyzed using GLP-1 EIA Kit (Sigma-Aldrich, USA), and DPP4 inhibitor was quantified by using HPLC method.

For the intestinal adhesion studies, the same protocol as for the permeability study was used, but instead of drug loaded NPs, AF488-conjugated NPs were added to the intestinal loop. After the incubation of the loops in Kreb's ringer buffer at 37 °C with gentle shaking for 3 h, they were washed twice with the buffer and small sections of the intestinal tissues were cut. The tissues were then fixed and stored in 4% paraformaldehyde at 4 °C. The tissues were embedded in paraffin, and small sections (5 µm) were cut and placed onto a glass slide. The sections were dewaxed and rehydrated, and stained with WGA-conjugated AlexaFluor 594 (10 µg/mL) for 10 min at room temperature. DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) (300 nM) was used to stain the nuclei of the cells by incubating with the cell monolayers for 4 min at room temperature. The tissues were again washed with buffer and the coverslips were mounted on the tissue sections. The slides were immediately observed under confocal fluorescence microscope.

4.7 *In vivo* study (IV)

4.7.1 Animal model

Animal experiments were approved by the Local Ethics Committee at the University of Porto and conducted under the guidelines and recommendations of FELASA and the European Directive 2010/63/EU. To evaluate the *in vivo* behavior of the H-CSUn NPs, adult Wistar rats (7 weeks old), weighing 180–200 g were purchased from Harlan Laboratories, Inc. The animals were housed in groups of 5 in standard conditions (temperature of 22 ± 2 °C, with relative humidity of 35% to 60% and 12 h light/dark cycles) with standard pellet food and water *ad libitum*. The rats were induced with T2DM prior to the main experiment. For this, the animals were fasted for 16 h, and then they were intraperitoneally (i.p.) injected with nicotinamide (120 mg/kg), followed by *i.p.* injection of streptozotocin (60 mg/kg) after 15 min. The T2DM induction was established by the raise in the blood glucose levels after 72 h [294-296].

4.7.2 *In vivo* evaluation after oral administration of the nanoparticles

The T2DM rats were fasted for 16 h before administration of the NPs. A number of formulations were administered via oral gavage, namely: (1) GLP-1 and DPP4 solution (2) empty H-CSUn NPs and (3) GLP-1 and DPP4 inhibitor loaded H-CSUn NPs. The amount of NPs administered were corresponding to 250 µg/kg of GLP-1 to the weight of the rats, using the gavage solution corresponding to 6.4 mg/mL of H-CSUn NPs. The NPs were dispersed in citrate buffer (pH 4) to avoid premature release of the drug in the stomach. Blood samples were withdrawn from the tail vein of the animals at different time intervals. Blood glucose levels were measured for 8 h using a glucometer, and the percentage of decrease in the blood glucose levels (%) were calculated using **Eq. (2)**.

$$\text{Decrease in blood glucose levels (\%)} = \frac{\text{AUC (Control)} - \text{AUC (Nanoparticles)}}{\text{AUC (Control)}} \times 100\% \quad \textbf{Eq. (2)}$$

In the **Eq. (2)**, AUC (control) is the area under the curve of the blood glucose levels after oral administration of the control solution of GLP-1 and DPP4 inhibitor or empty NPs without any drugs and AUC (Nanoparticles) indicates the area under the curve of blood glucose levels after oral administration of H-CSUn NPs with GLP-1 and DPP4 inhibitor. The plasma DPP4 levels were also measured with HPLC after extraction with acetonitrile.

5 Results and discussion

Particulate-based drug delivery systems with the ability to efficiently encapsulate and release proteins/peptides, protect against the harsh conditions of the gastric milieu, and enhance the intestinal drug permeability, hold a great potential for oral protein/peptide delivery. In this context, the potential of polymer, lipid and PSi based micro-/nanosystems as carriers for oral insulin and GLP-1 delivery were evaluated. Further modifications with different biofunctional moieties and encapsulation in the polymeric matrix were also performed, to impart several advantageous properties to the system, such as mucoadhesivity, cellular uptake, pH-responsivity and permeation enhancing property. Furthermore, the mechanism of uptake and transport of insulin from such systems with *in vivo* efficacy were also investigated.

5.1 Influence of surface chemistry and chitosan modification of PSi microparticles (I)

The properties of microparticles, such as surface charge, surface chemistry and presence of surface functional groups, can greatly influence the potential as drug delivery systems by affecting the ability to load drug molecules and/or their cellular interactions [22, 297]. Therefore, the focus of the first study was to evaluate the influence of the surface chemistry of PSi microparticles and the different approaches for chitosan surface modifications on insulin loading, interactions with intestinal cells, and permeability across intestinal cell monolayers. Hydrophobic AnnTHCPSi and slightly hydrophilic –COOH terminated AnnUnTHCPSi microparticles were compared, together with the evaluation of two different techniques of chitosan modifications of AnnUnTHCPSi: physical adsorption and chemical conjugation.

5.1.1 Insulin permeability and uptake across intestinal cell monolayers

The poor intestinal permeability of orally administered proteins and peptides is mainly due to the presence of the viscous mucus layer covering the epithelial cells and the TJs present between the adjacent intestinal epithelial [100]. Therefore, it is essential to evaluate the impact of the oral protein/peptide delivery systems on intestinal permeability of encapsulated protein/peptide. In this study, cell monolayers composed of enterocyte like Caco-2 and mucus producing HT-29 cells were used as a model for the intestinal epithelium. This model resembles intestinal epithelium more closely and overcomes the limitations of Caco-2 monolayers, such as tightness of the monolayers that resembles the colonic conditions, lack of mucus layer, comprised of only absorptive cells, and over-expression of P-glycoprotein [16, 292].

The permeability profiles of insulin from different microparticles are shown in **Figure 11A**. It can be clearly seen that bare AnnTHCPSi and AnnUnTHCPSi microparticles were able to enhance the permeability of insulin across the cell monolayers, with enhancement

ratios (ER) of 2.54 and 1.47, respectively, as compared to insulin solution. AnnTHCPSi microparticles showed better insulin permeation enhancing effect than AnnUnTHCPSi

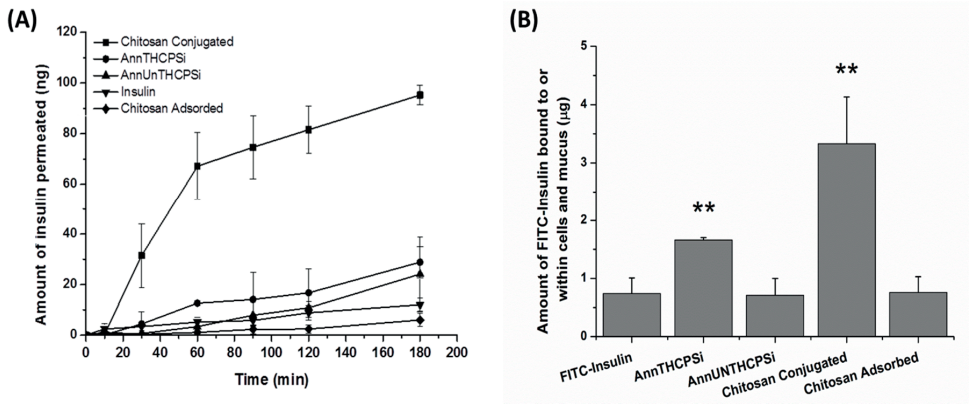


Figure 11 *(A) In vitro permeability profiles of insulin-loaded PSi microparticles across Caco-2/HT-29 co-culture monolayers. All the experiments were conducted from the apical (pH 6.5) to basolateral direction (pH 7.4) in HBSS–HEPES buffer at 37 °C. (B) Amount of FITC-insulin attached or taken up in Caco-2/HT-29 co-culture monolayers. Statistical analysis was made by the Student's t-test and all the data were compared to pure insulin. The level of significance was set at a probability of **$p < 0.01$. Error bars represent standard deviation (n = 3). Copyright © (2014) Elsevier B.V., reprinted with permission from publication (**I**).*

Furthermore, the highest insulin permeation across the monolayers was observed when chitosan was chemically conjugated to AnnUnTHCPSi microparticles, with an ER of 7.62. This augmented transport of insulin could be attributed to the presence of mucoadhesive chitosan present on the surface of the microparticles. Additionally, chitosan has also been reported to temporarily open the TJs between the intestinal epithelial cells thereby improving the intestinal insulin permeability [28, 244, 259]. Thus, modifying the surface of negatively charged PSi with chitosan renders the particles an additional advantage, which augments the insulin permeation across the intestinal cell monolayers. However, the chitosan adsorbed AnnUnTHCPSi microparticles did not demonstrate any permeation enhancing effect on insulin. This deteriorating effect can be due to the insulin loading, which occurs prior to chitosan coating, unlike chitosan conjugated AnnUnTHCPSi, where the loading was performed after the surface modification. The additional steps during chitosan adsorption and subsequent washing can lead to possible loss of insulin.

Uptake of insulin from these microparticles was also studied in the cell monolayers, which is shown in **Figure 11B**. The uptake results showed that the highest amounts of insulin associated with the cells and mucus were found with the chitosan-conjugated AnnUnTHCPSi, ($p < 0.01$), followed by the AnnTHCPSi microparticles, which corroborates with the permeability results. Overall, it was observed that the difference in surface chemistry of PSi microparticles influenced the insulin permeation across the cell monolayers, with the highest permeation effect observed for the chitosan-conjugated PSi microparticles.

5.1.2 Interaction of PSi microparticles with the intestinal cells

Possible interactions between the PSi microparticles and the intestinal cells (Caco-2 and HT29) were evaluated qualitatively by confocal fluorescence microscopy, **Figure 12**. The images clearly showed that there were minimal interactions between the unmodified particles and the cells, whereas the chitosan-modified PSi particles showed significant interactions with the cells. The poor interaction between the AnnTHCPSi and AnnUnTHCPSi microparticles can be explained by the negative surface charge, which can lead to repulsion from the negatively charged mucus layer and cell membranes [108]. Conversely, the chitosan-modified microparticles exhibited substantial interactions with the intestinal cells, which can also be attributed to the cationic charge and mucoadhesivity inherited by the presence of polycationic chitosan on the particle's surface [249]. The confocal images also showed higher cellular interactions with the chitosan-conjugated microparticles, as compared to the chitosan-adsorbed particles. The higher cell–particle interactions can be due to the presence of higher amounts of chitosan on the surfaces of the particles when chemically conjugated (11.7 ± 1.0 w-%), as compared to physical adsorption (1.1 ± 0.8 w-%).

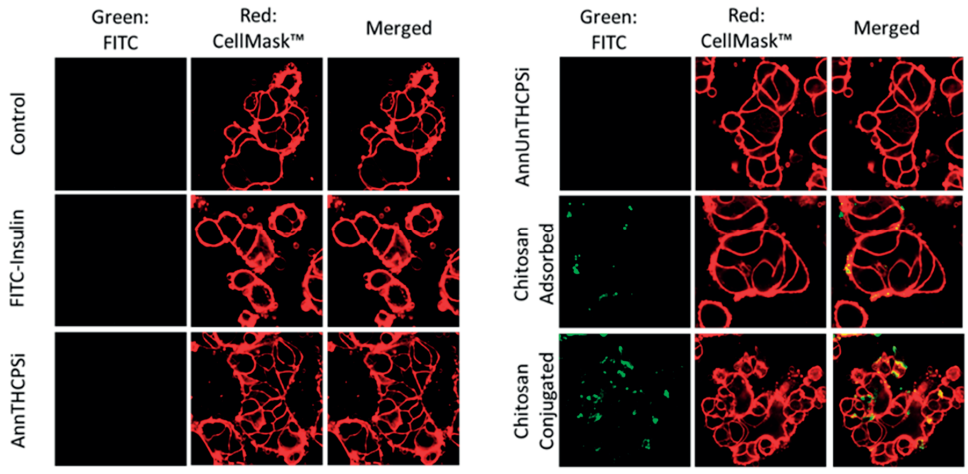


Figure 12 *Confocal microscope images of Caco-2/HT-29 co-culture monolayers treated with different PSi microparticles (400 µg/mL) loaded with FITC-labeled insulin after incubation with the cells at 37 °C for 3 h. Red: cell membranes stained with CellMask™ Orange; green: FITC-labeled insulin; yellow: co-localization of PSi microparticles and the cell membranes. Copyright © (2014) Elsevier B.V., reprinted with permission from publication (**I**).*

In conclusion, these results showed that the insulin permeation across intestinal cell monolayers could be enhanced by incorporating the insulin into the pores of the AnnTHCPSi microparticles. Additionally, the chitosan surface modification of AnnUnTHCPSi particles via chemical crosslinking showed the most efficient surface modification, leading to an enhanced intestinal insulin permeability, cellular uptake of insulin, and augmented cellular interactions.

5.2 Chitosan-modified nanoparticles as oral carriers for GLP-1 (II)

Different nanocarrier systems offer different advantages that can be beneficial for oral delivery of proteins and peptides. Thus, in the second study, a comparative analysis between nanocarriers composed of three different biomaterials, polymeric PLGA NPs, SLNs and UnTHCPSi NPs, were performed to evaluate their ability to deliver GLP-1 orally. The surface of all the nanosystems were modified with chitosan by physical adsorption (UnTHCPSi + CS, PLGA + CS, SLN + CS). These nanosystems were compared on the basis GLP-1 loading efficiency, *in vitro* release behavior, cellular interactions, and intestinal permeation of GLP-1.

5.2.1 GLP-1 encapsulation and *in vitro* release behavior

The physicochemical characterization of PLGA, SLNs and UnTHCPSi NPs presented a particle size of ~200 nm and a negative surface charge for all the NPs. After chitosan adsorption, a slight increase in the particle size was observed with conversion of the surface charge from negative to positive, indicating successful surface modification of the NPs. The GLP-1 loading evaluations showed that the UnTHCPSi NPs encapsulated the highest amount of GLP-1 with a loading degree of 17% (w/w), whereas the PLGA NPs and SLNs displayed a loading degree of only 0.17 % (w/w) and 0.18 % (w/w), respectively. The observed variances in the loading degree can be attributed to the loading technique used and the structure of these NPs. In the case of PSi NPs, the porous nature and the use of physical adsorption of the peptide into the pores of the NPs allowed the incorporation of high payloads [151]. However, the solid matrix based structure of PLGA NPs and SLNs limit the space available to encapsulate drug molecules. Additionally, the encapsulation of GLP-1 during the preparation steps also increases the probability of losing peptide during the multiple preparation step processes [298]. Nonetheless, after chitosan adsorption, a slight decrease in the loading degree was observed for all the NPs, which can be attributed to the loss of encapsulated GLP-1 during the coating process.

The nanocarriers were also evaluated based on the *in vitro* GLP-1 release behavior in simulated GI conditions. For this, the NPs were first exposed to pH 1.2 solution for 2 h mimicking the gastric conditions, and the next 4 h in FaSSIF to simulate the intestinal conditions. The GLP-1 release profiles of the unmodified and chitosan-modified NPs are shown in **Figures 13A** and **13B**, respectively. The release profiles showed that the SLNs did not retain the encapsulated GLP-1 in acidic conditions, with a burst release of 68%, and degradation of GLP-1 over time. The burst release observed for SLNs can be due to the hydrophilic nature of the peptide, which tends to accumulate at the o/w interface during the fabrication process [230, 299, 300]. UnTHCPSi and PLGA NPs sustained the release of GLP-1 to some extent in the acidic conditions, with burst GLP-1 release in simulated intestinal conditions. This biphasic GLP-1 release behavior from the PLGA and UnTHCPSi can be explained by the nature of their interactions with GLP-1 (pI = 5.4). At acidic conditions, GLP-1 carries a net positive charge and tends to interact strongly to the negatively charged particles. However, the charge of GLP-1 in basic intestinal conditions is negative, which leads to minimal interactions with the NPs due to charge repulsion [196,

301]. The chitosan coated NPs depicted a more sustained release of GLP-1 in both conditions, despite similar release patterns (**Figure 13B**). These results are in accordance with other reports where chitosan has been shown to decrease the burst release of the drugs encapsulated in NPs [299].

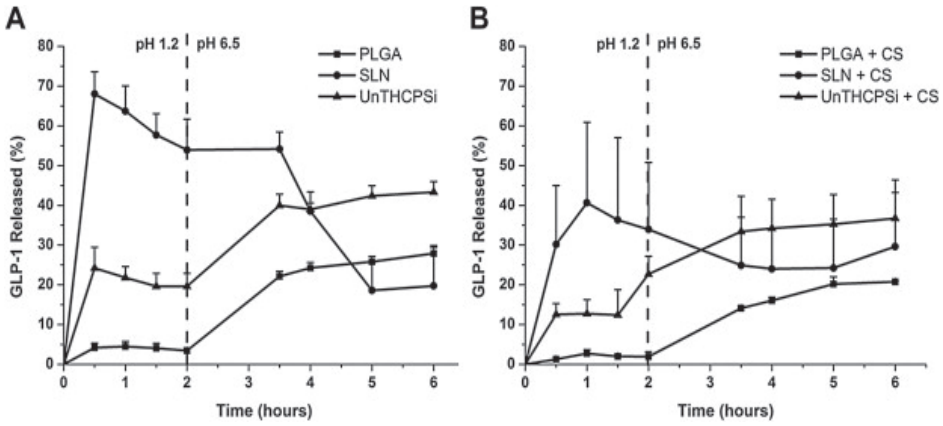


Figure 13 *Release profiles of GLP-1 from (A) PLGA NPs, SLNs and UnTHCPSi NPs and from (B) PLGA + CS, SLN + CS and UnTHCPSi + CS NPs. The percentages of peptide released from uncoated NPs were compared with their corresponding CS-coated NPs at the same time points, and compared with the other NPs. All experiments were conducted at pH 1.2 for the first 2 h and in FaSSIF (pH 6.5) for the next 4 h at 37 °C and 100 rpm. Error bars represent the mean ± SD (n = 3). Copyright © (2014) Elsevier B.V., reprinted with permission from publication (**II**).*

Overall, it was observed that PLGA and UnTHCPSi NPs averted the release of GLP-1 in gastric conditions to some extent, with higher GLP-1 release at intestinal conditions. Furthermore, it was also shown that after chitosan coating, the NPs suppressed the premature release of GLP-1 in gastric conditions and released it in the intestinal medium.

5.2.2 Intestinal transport of GLP-1 from chitosan-coated nanoparticles

From the release studies, it was evident that the chitosan coated NPs improved the GLP-1 release behavior, as compared to the unmodified NPs. Hence, the permeability of GLP-1 across cellular intestinal monolayers was only assessed for the chitosan-modified NPs. For this, triple co-culture cell monolayers were used as the intestinal cell model. The monolayers comprised enterocytes (Caco-2 cells), mucus-producing goblet cells (HT29-MTX), and M cells, which were induced from Caco-2 cells in the presence of Raji B cells [293]. All the three cell types together formed a monolayer with TJs that resemble the intestinal epithelium more closely [293, 302].

The GLP-1 permeability profiles from the different chitosan modified nanosystems are shown in **Figure 14A**. Among the different systems, free GLP-1 solution exhibited the highest permeation across the cell monolayers, which can be explained by the presence of

high amounts of free peptide in the apical side as compared to the NPs, where only partial amount of total GLP-1 was released. Additionally, the release of GLP-1 from the different NPs would have been even more sustained in HBSS–HEPES as compared to FaSSIF, because of the absence of natural surfactants, such as bile salts, in the buffer solution. Comparing the NPs, the PSi NPs showed the highest GLP-1 permeation, followed by SLNs and PLGA NPs. These results are in accordance with the *in vitro* release studies (**Figure 13**) that displayed the maximum sustained GLP-1 release behavior from PLGA + CS NPs, thus having the least amount of peptide available to permeate across the cell monolayers. Nevertheless for SLN + CS, although GLP-1 showed higher permeation effect as compared to PLGA + CS NPs, the GLP-1 release profile must be taken into consideration as there is a loss of ~ 40% GLP-1 in the gastric conditions before reaching the intestinal environment.

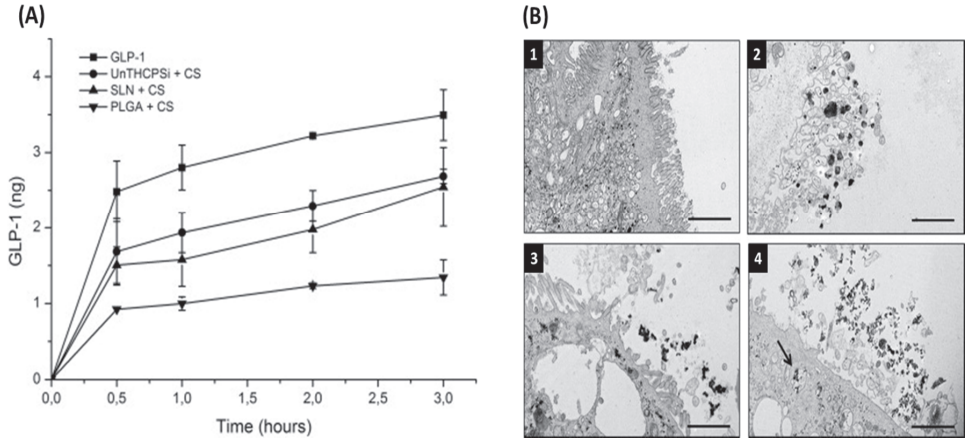


Figure 14 *(A) In vitro cumulative permeability profiles of GLP-1 from CS-coated NPs across Caco-2:HT29-TX: Raji B co-culture monolayers. All experiments were conducted from the apical (pH 6.5) to the basolateral direction (pH 7.4) in HBSS at 37 °C. Error bars represent mean ± SD (n = 3). (B) TEM images of flat embedded ultrathin sections after permeability showing control cells (1), PLGA + CS NPs (2), SLN + CS NPs (3), and UnTHCPSi + CS NPs (3). Copyright © (2014) Elsevier B.V., reprinted with permission from publication (**II**).*

The GLP-1 transport observed can be attributed to the presence of mucoadhesive chitosan present on the surface of the NPs. The cell monolayers were examined under TEM to visualize any possible interactions with the NPs. In the TEM image in **Figure 14B**, it was possible to observe that the chitosan modified NPs were closely interacting with the intestinal epithelium. This interaction with the intestinal cell monolayer can be due to the positive charge of the particles imparted by the chitosan surface modification. These results are in agreement with other studies in the literature, which have exhibited similar increase in interactions between the positive surfaces and the negative charge moieties of the cell membrane leading to enhanced cellular interactions [156, 303]. The mucoadhesive properties of the NPs and enhanced GLP-1 permeation across the cell monolayers can

improve the GLP-1 intestinal absorption, and thus, can possibly improve its oral bioavailability [304].

Overall, the results of this study showed that for oral GLP-1 delivery, each nanosystem offered different advantages: high loading and permeation enhancing effect for PSi-based NPs, sustained drug delivery for PLGA NPs and comparatively better GLP-1 permeability for SLNs.

5.3 Multistage pH-responsive polymer-PSi delivery systems for GLP-1 and DPP4 inhibitor (III–IV)

In addition to the physical barrier posed by the intestinal membrane, the biochemical barrier comprising the highly acidic gastric environment and abundant digesting enzymes present along the GI tract, may act as one of the major hurdles for oral delivery of proteins/peptides [300]. Therefore, with the aim to overcome these hindrances, a multifunctional site-specific dual protein-drug delivery nanosystem was developed to co-deliver GLP-1 and DPP4 enzyme inhibitor orally. Briefly, the nanosystem comprised of chitosan-modified PSi NPs (CSUn) with GLP-1 loaded in their porous structure. The NPs were then coated with a pH-responsive polymer using AFR technology, which prevented premature release and degradation of GLP-1 in the gastric conditions, and provided a site for encapsulation of DPP4 inhibitor [21]. In this study, undecylenic acid modified thermally hydrocarbonized PSi (abbreviated as UnPSi), were modified with chitosan by chemical crosslinking, and then coated with an enteric polymer, HPMCAS-MF (H-CSUn). This multidrug nanosystem was evaluated based on its physicochemical properties, *in vitro* and *ex vivo* intestinal permeability, and *in vivo* performance.

5.3.1 pH-responsive behavior of the multistage polymer–PSi nanocomposite

Firstly, the surface morphology of the H-CSUn NPs and polymeric NPs without PSi were evaluated using HR-SEM (**Figure 15A**). The polymeric NPs displayed spherical shape with smooth surface, whereas the H-CSUn NPs showed an uneven surface indicating a successful encapsulation of CSUn NPs in the polymeric matrix. The successful coating was further confirmed by evaluating the pH-responsivity of the system using TEM, size of the nanocarriers, and the drug release profiles. From the TEM images (**Figure 15B**), it can be clearly observed that the NPs remained intact in acidic pH until 1 h, whereas in pH 6.0 and 6.8,the polymeric coat started to dissolve in the first 10 min and completely dissolved after 1 h, releasing the encapsulated CSUn NPs. Furthermore, the pH responsiveness of the nanosystem was confirmed with the decrease in the size of the NPs from 830 to 203 nm when the pH was changed from 1.2 to 6.8 (**Figure 15C**). The pH responsivity of the nanosystem can be attributed to pH dependent solubility of HPMCAS-MF, which only dissolved at pH $\geq$ 6 [279].

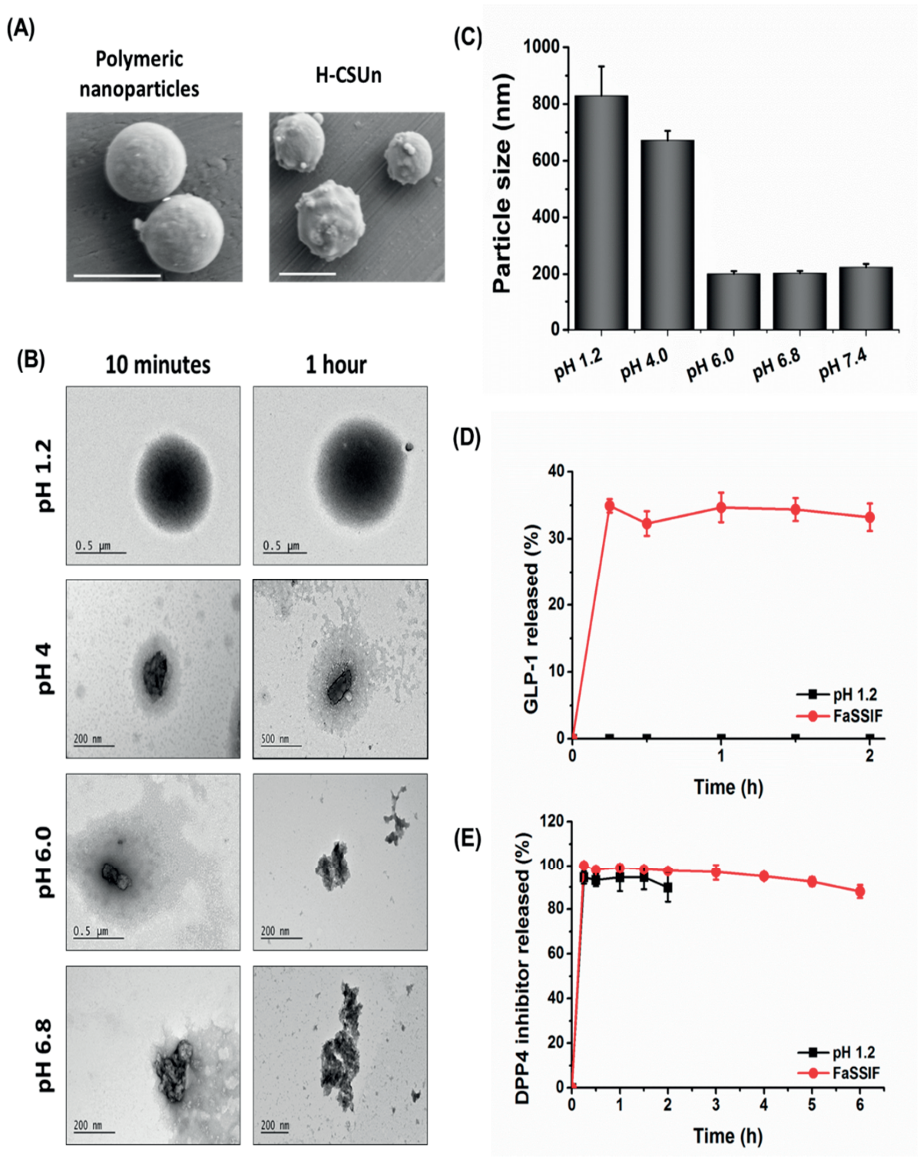


Figure 15 *(a) HR-SEM images of the morphology of HPMCAS-MF polymeric NPs and HPMCAS-MF coated CSUn NPs (scale bar = 500 nm). (b) TEM images showing the pH-responsive dissolution behavior of the H-CSUn NPs after incubation at different buffer solutions of pH values (1.2, 4, 6 and 6.8) for 10 min and 1 h. Dissolution initiated at pH ≥ 6.0, wherein the polymeric coat start to dissolve in the first 10 min and then was completely dissolved in 1 h. (c) Particle size of the H-CSUn NPs after incubating with different buffers at pH values of 1.2, 4, 6.0, 6.8 and 7.4. Data is shown as mean ± SD (n = 3). In vitro cumulative drug release profiles of (d) GLP-1 and (e) DPP4 inhibitor from H-CSUn at pH 1.2 and FaSSiF (pH 6.8) at 37 °C. Data is shown as mean ± SD (n = 3). Copyright © (2015) Elsevier B.V., reprinted with permission from publication **(III)**.*

The *in vitro* drug release behavior was also studied for these systems in two different release media: pH 1.2 solution for 2 h and in FaSSiF (pH 6.8) for 6 h, mimicking its passage in the GI tract. For GLP-1 (**Figure 15D**), the NPs were able to successfully retain the loaded GLP-1 at pH 1.2 with no detectable amounts of GLP-1 released in the gastric conditions. Nonetheless, the same nanosystem showed a burst GLP-1 release (~37%) in FaSSiF medium. This pH-dependent release of GLP-1 was due to the dissolution of the enteric coat at pH>6, as discussed above. Nonetheless, the release of GLP-1 from the NPs was incomplete, which could be explained by the strong hydrophobic interactions between the NPs and the peptide, slower degradation rate of UnPSi, and irreversibly adsorbed peptide on the pore walls [22, 199]. This delayed drug release profile avoids the premature release and degradation of the peptide in the stomach, and increases the available amount of GLP-1 for absorption in the intestine. In the case of DPP4 inhibitor release (**Figure 15E**), a burst release of DPP4 inhibitor was observed in both gastric and intestinal conditions, which suggested the presence of the drug on the surface of the NPs [305]. This can be due to the high aqueous solubility of the inhibitor, nano-size of the particles, and low drug entrapment [217, 306]. Nevertheless, the DPP4 inhibitor has been shown active after oral administration, thereby making it therapeutically available despite of the premature release in the stomach [81, 82].

In summary, the results above demonstrated successful formation of pH sensitive coatings on CSUn NPs, which enabled site-specific release of GLP-1 only in the intestine.

5.3.2 Cellular viability study

Cytotoxicity of the NPs was evaluated in AGS cells, a gastric epithelial cell line originated from human gastric adenocarcinoma for 3 h, and in intestinal cell lines, Caco-2 and HT29-MTX cells, for 12 h. The evaluation of the cellular viability in these three cell lines for different time points represents the maximum transit times in the GI tract when the nanocarriers are orally administered. Moreover, the several concentrations tested also avail information regarding any component or concentration-dependent toxicity. The cellular toxicity of UnPSi, CSUn and H-CSUn NPs were tested in different concentrations (25–400 μg/mL).

The cellular toxicity results for all the NPs are shown in **Figure 16**. The bare UnPSi NPs showed a concentration-dependent toxicity in the intestinal cell lines; however, the cell viability remained higher than 80% in the gastric cells. Moreover, the CSUn NPs showed improved cytocompatibility with cell viability values above 80% for all the three cell lines tested. The enhanced cytocompatibility can be attributed to the chitosan, which has been previously reported to be biocompatible and non-toxic [244, 247]. Comparing the cell lines, higher cytotoxicity was observed in Caco-2 cells as compared to the HT29-MTX cells, which can be explained by the presence of a mucus layer on HT29-MTX cells, thus avoiding close interactions of the NPs with the cell surfaces [292]. The final multistage H-CSUn NPs also showed high cellular viability for all the tested concentrations and cells, thus demonstrating the fact that the polymeric coating did not have any harmful effect on cytocompatibility. In addition, HPMCAS-MF, the pH responsive polymer used in this study, has already been approved by the FDA [277].

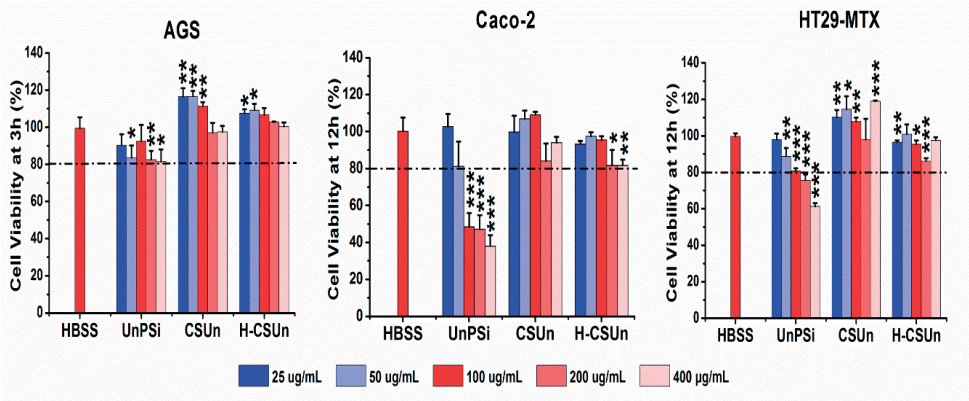


Figure 16 *Cell viability of the GI cells exposed to PSi NPs (modified and unmodified with chitosan) assessed by the CellTiter-Glo® luminescence assay. The ATP contents of AGS (3 h), Caco-2 (12 h) and HT29-MTX (12 h) cells after the incubation with different PSi NPs at different concentrations was investigated. The NPs were incubated with the cells at 37 °C. Statistical analyses were made by one-way analysis of variance (ANOVA) with Bonferroni post-test. All the data sets were compared to the negative control HBSS–HEPES buffer. The levels of significance were set at probabilities of *p < 0.05, **p < 0.01, and ***p < 0.001). Data is shown as mean ± SD (n = 3). Copyright © (2015) Elsevier B.V., reprinted with permission from publication* ***III****).*

5.3.3 Enhanced intestinal permeability of GLP-1 across cell monolayers

The efficiency of the multistage nanosystems to enhance the intestinal permeability of GLP-1 and DPP4 inhibitor was evaluated using a triple co-culture model based on Caco-2, HT29-MTX and Raji B cells, as described in *Section 4.5.5*. The permeability profiles and apparent permeability (P_{app}) coefficients for the GLP-1 and DPP4 inhibitor are shown in **Figure 17A–D**. The GLP-1 permeability profiles (**Figure 17A**) showed significantly enhanced GLP-1 transport when encapsulated into the H-CSUn NPs, which was also shown by a significant increase in the P_{app} ($p < 0.01$) (**Figure 17B**). The enhanced GLP-1 permeability can be explained by the presence of mucoadhesive and permeation enhancing chitosan on the surface of the NPs that augmented the passage of peptides to the basolateral side [28, 307, 308]. When the DPP4 inhibitor was included in the nanosystem (designated as H-CSUn (D)), the GLP-1 permeation was further enhanced with significant increase in the P_{app} value ($p < 0.001$). This increase of the GLP-1 transport across the cell monolayers can be attributed to the presence of DPP4 inhibitor in the system. DPP4 inhibitor is responsible for the inhibition of the DPP4 enzyme that is accountable for rapid degradation of GLP-1, leading to a very short half-life of GLP-1 of ~2 min [59, 72, 309]. This short half-life of GLP-1 is one of the major limiting factors for its use in T2DM therapy, due to which a continuous administration of GLP-1 is required. Therefore, addition of DPP4 inhibitor in the nanosystem greatly increases the amount of active GLP-1 permeation across the intestinal monolayer. In addition, DPP4 inhibitor has also been reported to have beneficial effects on the treatment of T2DM in humans, preventing weight gain, potentiation of β–cell

function, and suppression of plasma glucagon [72]. The permeability of DPP4 inhibitor across the intestinal cell monolayers was also studied (**Figure 17C**). There were no significant changes observed in the permeability profiles or P_{app} (**Figure 17D**) for both the pure drug solution and H-CSUn (D).

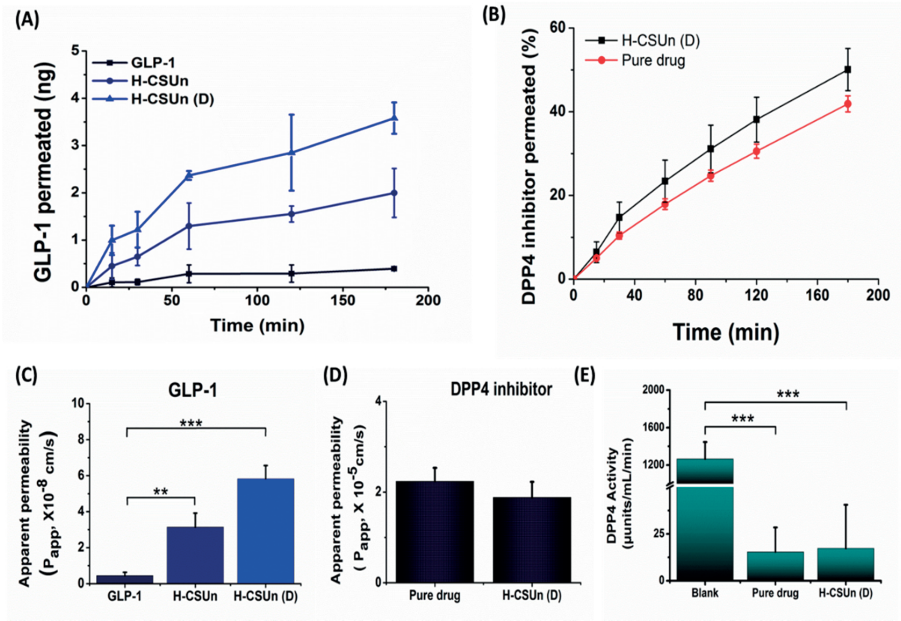


Figure 17 *In vitro permeability profiles of (a) GLP-1 and (b) its apparent permeability (Papp) coefficient. In vitro permeability profiles of (c) DPP4 inhibitor and (d) its Papp coefficient. Permeability was evaluated across the Caco-2/HT-29/Raji B triple co-culture monolayers. The experiments were performed from the apical-to-basolateral chamber in HBSS–HEPES buffer (pH 7.4) at 37 °C. Data is shown as mean ± SD (n = 3). One-way ANOVA was performed with the level of significance set at a probability of ***p < 0.001. (e) DPP4 enzyme activity studied in the Caco-2/HT29-MTX/Raji B triple co-culture monolayers in the presence of DPP4 inhibitor solution (pure drug) and from the H-CSUn NPs (H-CSUn (D)). One-way ANOVA was performed with the level of significance set at a probability of ***p < 0.001. Copyright © (2015) Elsevier B.V., reprinted with permission from publication (**III**).*

Furthermore, to demonstrate the involvement of DPP4 inhibitor in the enhancement of the GLP-1 permeability, the activity of the DPP4 enzyme in the cell monolayers with and without the inhibitors was evaluated. The enzyme activities of the cell monolayers incubated with buffer, pure inhibitor solution or H-CSUn (D) are shown in **Figure 17E**. The results showed significant reduction in the enzyme activity when treated with the DPP4 inhibitor, either as a solution ($p < 0.001$) or as NPs ($p < 0.001$). Thus, it can be concluded that the DPP4 inhibitor is a critical component of the nanosystem to promote the absorption of GLP-1 across the intestinal epithelium in an intact form. Additionally, the enzyme activity study also showed that the activity of DPP4 inhibitor was preserved throughout the coating process in the AFR experiments.

5.3.4 *Ex vivo* GLP-1 and DPP4 inhibitor permeability

The permeability of GLP-1 and DPP4 inhibitor from the H-CSUn NPs was further investigated *ex vivo* using ligated loops of freshly excised rat intestine tissues [126]. From the GLP-1 permeability profiles shown in **Figure 18A**, it can be observed that the NPs significantly enhanced the intestinal permeability of GLP-1, with 3.9-fold higher amounts of GLP-1 permeated after 3 h as compared to the control (GLP-1 + DPP4 inhibitor solution) ($p > 0.05$). The result indicated absorption enhancement of GLP-1, which can be attributed the presence of chitosan and DPP4 inhibitor in the nanosystem. Furthermore, the permeability profile of DPP4 inhibitor (**Figure 18B)** showed that the difference between the control and the H-CSUn NPs was insignificant. The *ex vivo* GLP-1 and DPP4 inhibitor permeability results are in accordance with the results obtained from the *in vitro* cell monolayer studies (*Section 5.3.3*).

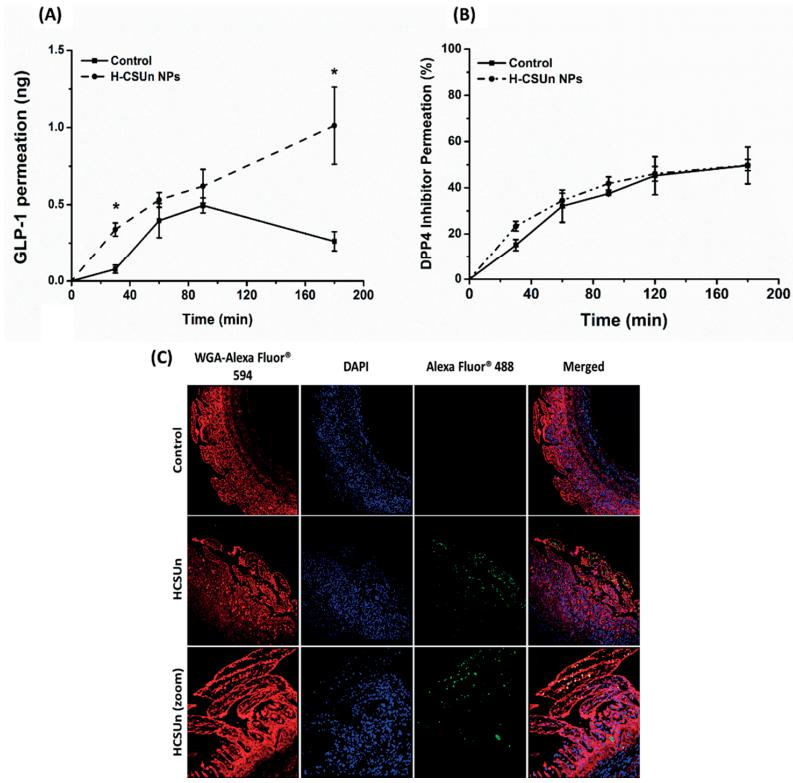


Figure 18 *Cumulative (A) GLP-1 and (B) DPP4 inhibitor transported across the rat intestine tissues in an ex vivo ligated intestinal loop at 37 °C in oxygenated Krebs Ringer buffer. The level of significance were set at a probability of *p < 0.05. Data shown as mean ± SEM (n = 3). (C) Confocal microscopy images of the intestinal mucoadhesion study with H-CSUn NPs (scale bar = 100 µm). The NPs were incubated with the ligated intestinal loop at 37 °C for 3 h. The tissues were embedded in paraffin and cut in 5 µm sections. The tissues' sections were then dewaxed, rehydrated and stained. Red: WGA AlexaFluor 594; green: AF488-conjugated NPs; blue: DAPI; and yellow: co-localization of mucus and NPs* ***(IV)****.*

In addition to the permeability study, the ability of the NPs to interact with the rat intestinal epithelium was also evaluated using confocal microscopy, as shown in **Figure 18C**. The NPs demonstrated good interactions with the surface of the intestinal epithelia after 3 h incubation. The observed intestinal adhesion can once again be attributed to the presence of the mucoadhesive chitosan in the NPs [249]. Similar results were also observed with H-CSUn NPs interacting with the intestinal cell lines (**III)**. Overall, from the *ex vivo* studies it could be established that the H-CSUn NPs efficiently enhanced the GLP-1 permeability across the intestinal epithelia.

5.3.5 *In vivo* effect of the nanocarrier

After demonstrating the cytocompatibility, enhanced interaction with the intestinal epithelium and augmented intestinal permeability of GLP-1, the efficacy of H-CSUn NPs was further evaluated *in vivo* in diabetic rats. The NPs were administered to the diabetic rats by oral gavage, and the blood glucose levels were evaluated, as shown in **Figure 19A**. After oral administration, the H-CSUn NPs showed notable anti-hyperglycemic effect as compared to the control (GLP-1 and DPP4 inhibitor solution) at 30, 60 and 120 min ($p < 0.05$).

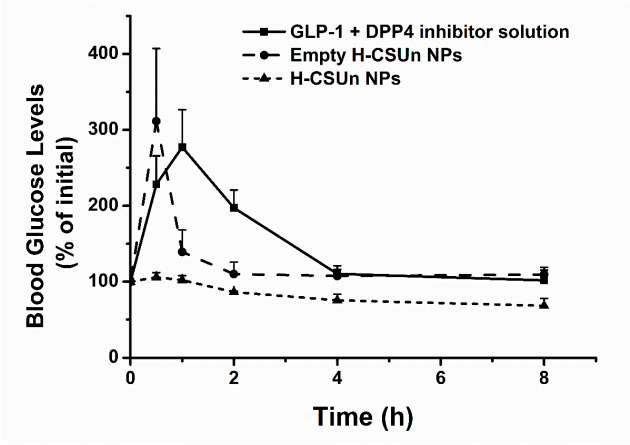


Figure 19 *(A) Blood glucose levels in diabetic rats after oral administration of H-CSUn NPs at the dose of GLP-1 of 250 µg/kg in T2DM rat. Data shown as mean ± SEM (n = 3) (**IV**).*

To further understand the results clearly, a decrease in blood glucose levels, decrease in blood glucose levels (%) was also calculated using **Eq. (2)**. The decrease in blood glucose levels (%) of the H-CSUn NPs was calculated to be 45% and 27 % when compared to the control and the empty NPs, respectively. These values demonstrated that the blood glucose levels were markedly lowered after oral administration of the H-CSUn NPs as compared to the controls. The decrease in the blood glucose levels can be attributed to the powerful insulinotropic peptide, GLP-1, which stimulates the pancreatic insulin secretion and release in a glucose-dependent manner, and the inhibition of the degradation of GLP-1 by the presence of the DPP4 inhibitor [59, 65, 68]. Furthermore, the advantageous properties of

the multistage nanosystem to protect the GLP-1 in the harsh gastric conditions with burst release only in the intestine, enhanced interaction with the intestinal membrane and augmented GLP-1 permeability had a combined beneficial effect to deliver the GLP-1 and DPP4 inhibitor simultaneously. In summary, the H-CSUn NPs exhibited potential as a nanocarrier system for oral delivery of GLP-1 and DPP4 inhibitor, simultaneously.

5.4 Influence of L-cysteine and CPP functionalization of chitosan-modified PSi nanoparticles on intestinal permeability of insulin (V)

The ability of chitosan modified PSi based micro- and nanoparticles to enhance the intestinal permeability of proteins and peptides has already been established. Thus, this part of the dissertation aimed at potentiating the permeation enhancing effect of chitosan-modified NPs by further surface modifications. For this, L-cysteine (CYS-CSUn) or CPP (CPP-CSUn) were supplemented to the surface of CSUn NPs. These nanocarriers were characterized based on the different physicochemical properties, insulin loading and *in vitro* insulin release behavior. The effect of these surface modifications on the insulin intestinal permeability was also evaluated along with the study of insulin uptake and transport mechanism studies from these nanocarriers across the triple co-culture intestinal cell model.

5.4.1 Insulin permeability across the intestinal epithelium

The successful surface modification of the NPs to form CSUn, CYS-CSUn and CPP-CSUn was confirmed by the physicochemical characterization, which is explained in detail elsewhere (**V**). L-cysteine was used to add a thiol groups to the surface of the CSUn NPs, as thiolated NPs have been shown to have advantageous influence on the mucoadhesivity and permeation enhancement [263, 310, 311]. In addition, oligoarginine CPP was selected for further surface functionalization of the NPs as the positively charged structure has been shown to enhance the cellular interactions and translocation of the NPs [20, 273].

The ability of these NPs to enhance the intestinal permeability of insulin was evaluated across the triple co-culture intestinal model, as described above (*Section 4.5.5*). The permeability profiles and the enhancement ratio of the P_{app} coefficients are shown in **Figures 20A** and **20B**, respectively.

A significant increase in the insulin permeability was observed after modification in the following order: CYS-CSUn > CPP-CSUn > CSUn. A 17- and 12-fold enhancement in the P_{app} coefficient were observed for the CYS-CSUn and CPP-CSUn, respectively, when compared to CSUn. The presence of thiol groups on the surface of the CYS-CSUn NPs increased the interactions with the mucin glycoproteins present in the intestinal epithelia, thereby enhancing the mucoadhesivity of the NPs and the NP–cell interactions [30]. Additionally, the thiol groups have been reported to stimulate the opening of the TJs of the intestinal cells, thus promoting the insulin permeation across the cell monolayers [265, 267]. In the case of CPP-CSUn, the ability of the CPP to interact with the glycosaminoglycans present on the cell's surface and enter into the cells via endocytic pathway could be the

reason for the observed enhanced permeation [20, 271]. Therefore, it could be concluded that the permeation enhancing effect of CSUn can be significantly improved by the modification of CSUn NPs with either L-cysteine or CPP.

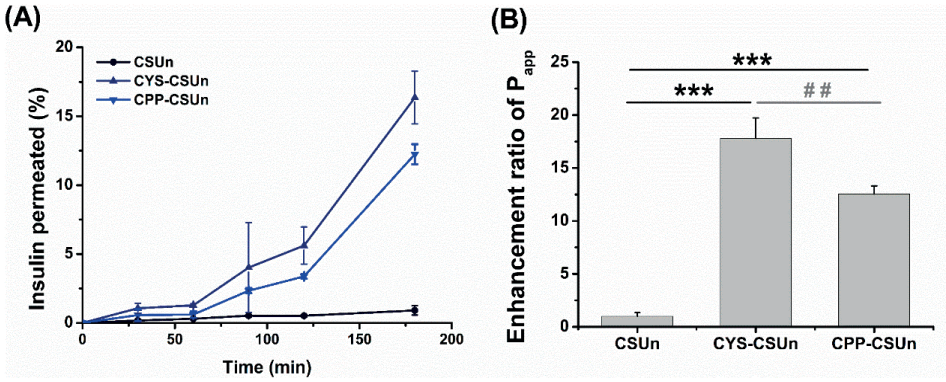


Figure 20 *(A) In vitro permeability profiles of insulin from different NPs (CSUn, CYS-CSUn, and CPP-CSUn) across the Caco-2/HT-29/Raji B triple co-culture cell monolayers. (B) The enhancement ratios of the P_{app} coefficients of insulin from the different NPs. The levels of significance were set at probabilities of $^{***}p < 0.001$ for comparison between CSUn and CYS-CSUn NPs, and between CSUn and CPP-CSUn NPs, and $^{\#\#}p < 0.01$ for comparison between CYS-CSUn and CPP-CSUn NPs. Data shown as mean ± SD ($n \geq 3$). Copyright © (2016) John Wiley & Sons, Inc., reprinted with permission from publication (V).*

5.4.2 Cell–nanoparticle interactions

After demonstrating the permeation enhancing effect of the NPs, they were further analyzed for the interactions with the intestinal cell lines. The interactions were studied qualitatively using confocal fluorescence microscopy imaging (**Figure 21A**) and TEM (**Figure 21B**), and quantitatively using flow cytometry (**Figure 21C**). The qualitative studies were performed with the triple co-culture intestinal cell monolayer (*Section 4.5.5*). In the confocal fluorescence microscopy images, it was demonstrated that although the CSUn NPs were interacting with the mucus layer, much higher interactions were observed for the CYS-CSUn and CPP-CSUn, which could be seen by the large amounts of co-localized NPs with the mucus. Similar observations were also made with TEM images, where the CYS-CSUn and CPP-CSUn showed higher interactions compared to the CSUn NPs. These qualitative results were further confirmed by quantitative flow cytometry analyses, as shown in **Figure 21C**. The flow cytometry results indicated that the percentage of positive events was significantly higher for CYS-CSUn and CPP-CSUn NPs as compared to CSUn, which corroborated the qualitative studies.

For CYS-CSUn NPs, the thiol groups immobilized on the surface forms disulfide bonds with the mucin glycoproteins, thus, leading to mucoadhesion [265, 267]. In the case of CPP-CSUn NPs, the enhanced interactions with the mucus layer of the intestinal cells can be attributed to the presence of positively charged oligoarginine CPP on the surface of the NPs

[20, 271]. The results obtained from this study are in accordance with the insulin intestinal permeability study (*Section 5.4.1*), where the CYS-CSUn and CPP-CSUn showed further augmentation of insulin permeation as compared to the CSUn NPs.

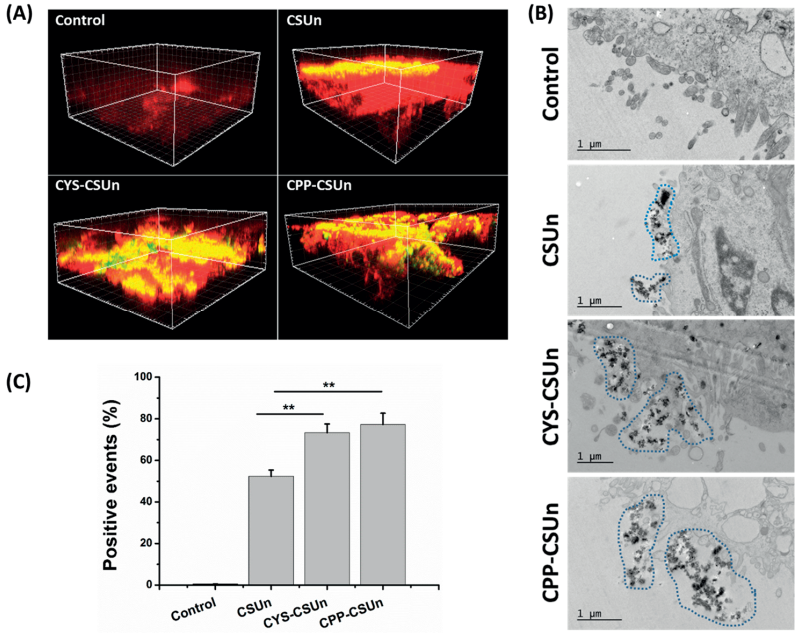


Figure 21 *In vitro NP– cell interaction study after incubating the NPs with the cells at 37 °C for 3 h. (A) 3D confocal fluorescence microscope images of the cell monolayers interacting with different NPs (Red: WGA AlexaFluor 594; Green: AF-488 NPs; and Yellow: Co-localization of mucus and NPs). (B) TEM images of flat embedded ultrathin sections of cell monolayers interacting with different NPs. (C) Flow cytometry quantitative analysis of the Caco-2/HT29-MTX co-culture cells interacting with different NPs. The levels of significance were set at the probability of **$p < 0.01$ between the CSUn and CYS-CSUn NPs or CPP-CSUn NPs. Copyright © (2016) John Wiley & Sons, Inc. reprinted with permission from publication (**V**).*

5.4.3 Insulin transport and cellular uptake mechanisms

The insulin permeation and cellular interaction studies showed significant augmentation of the intestinal insulin permeability after the L-cysteine and CPP modifications of the CSUn NPs. However, the transport and uptake mechanisms involved for such NPs have not been well studied before. Therefore, in this study, the possible uptake and transport mechanisms of insulin-loaded NPs across the triple co-culture cell monolayers were investigated in detail.

There are several possible drug intestinal uptake/transport mechanisms at the intestinal level, such as paracellular, active transport, adsorptive endocytosis, and electrostatic interactions (**Figure 5)**. To examine the possible involvement of these mechanisms, insulin-loaded NPs were incubated with the cell monolayers in the presence of different uptake

inhibitors, followed by insulin association and transport studies across the cell monolayers. The insulin associated with the monolayers is shown as the amount of insulin per mg of the total protein content of the cell monolayers and plotted as the percentage with respect to the control without any inhibitors (**Figure 22A**). The insulin transported in the presence of inhibitors is shown in **Figure 22B** as a percentage of the control.

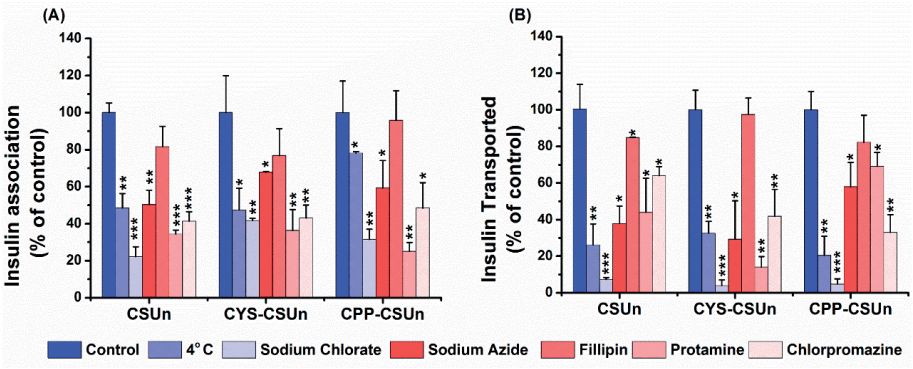


Figure 22 *(A) Uptake and (B) transport of insulin from the different NPs across the triple co-culture cell monolayers in different conditions. All the data sets were compared to the respective controls. The levels of significance were set at probabilities of *p < 0.05, **p < 0.01, and ***p < 0.001. Data are shown as mean ± SD (n = 3). Copyright © (2016) John Wiley & Sons, Inc., reprinted with permission from publication (V).*

The involvement of active transport was disclosed, as a significant decrease in insulin uptake and transport were seen in the presence of sodium azide and at 4 °C, which have been shown to inhibit the active transport in the cells [119]. Likewise, pre-treatment with sodium chlorate exhibited the most significant reduction in the uptake and transport of insulin for all the tested NPs. Sodium chlorate has been shown to inhibit the electrostatic interactions between the NPs and the glycocalyx of the cells, inhibiting the glycosaminoglycan sulfation [119, 312]. Moreover, the electrostatic interactions between the NPs and the integrin on the cell surfaces have been reported to be the primary step to stimulate the cascade of reactions for opening the TJs [253]. Additionally, the inhibition with protamine and chlorpromazine also reduced the insulin uptake and transport across the cell membrane significantly, suggesting the involvement of adsorptive endocytosis and clathrin-mediated endocytosis. Protamine is responsible for the inhibition of adsorptive endocytosis, and chlorpromazine has been stated to disrupt the assembly and disassembly of clathrin, thus hindering the receptor recycling involved in the clathrin-mediated endocytosis [313, 314]. However, the presence of fillipin inhibitor did not show any significant changes in the transport and uptake of insulin, suggesting the absence in the involvement of caveolae-mediated endocytosis. Fillipin inhibits the caveolae-mediated endocytosis by precipitating cholesterol that damages the caveolae structure [315].

Overall, from this study it was observed that there is probable involvement of several pathways, such as active transport, electrostatic interactions, adsorptive and the clathrin-mediated endocytosis process, in the uptake and transport of insulin from these modified and insulin-loaded PSi-based NPs.

6 Conclusions

In this dissertation, PSi-based micro and nanoparticulate systems were developed as oral carrier systems for insulin and GLP-1, and were evaluated based on their ability to enhance the intestinal permeability of the encapsulated drugs.

In the first study, the influence of different surface chemistries and chitosan surface modification methods of PSi microparticles on insulin permeability across intestinal cell monolayers were evaluated. Comparing different surface chemistries, AnnTHCPSi microparticles showed significantly higher insulin permeability and uptake as compared to AnnUnTHCPSi microparticles. The highest insulin permeation and uptake was observed for chitosan-conjugated AnnUnTHCPSi microparticles, which was elucidated by high interactions with the intestinal cells, as clearly shown in the confocal and TEM images.

In the next study, three different nanosystems fabricated from three different biomaterials, polymer, lipid and PSi, were evaluated as potential oral GLP-1 delivery systems. Chitosan coating of the NPs greatly improved these nanosystems by sustaining the release of GLP-1, enhancing the cellular compatibility and interactions, and augmenting the GLP-1 transport across the intestinal cellular monolayers. However, among the three nanosystems, chitosan-coated PSi NPs showed the best performance with the highest GLP-1 loading, sustained GLP-1 release behavior and the highest amount of GLP-1 permeated, thus demonstrating itself as a potential carrier for oral GLP-1 delivery.

Next, taking advantage of the potential of PSi NPs and the chitosan surface conjugation to overcome the physical intestinal barriers, an advanced multistage pH-responsive polymer/PSi nanocomposite was developed using AFR technology for dual-drug delivery of GLP-1 and DPP4 inhibitor. In addition to the mucosal and intestinal barriers, this nanosystem was also aimed at overcoming the biochemical barriers in the GI tract by protecting the encapsulated GLP-1 from the harsh gastric conditions and prolonging the GPL-1 half-life by simultaneous delivery of DPP4 inhibitor from the same nanosystem. This dual-drug delivery system exhibited enhanced GLP-1 permeability across both *in vitro* intestinal cell monolayers and *ex vivo* rat intestinal tissues. This augmented permeability could be attributed to high interactions with the intestinal epithelium and inhibition of the DPP4 enzymes present in the intestinal epithelium. Moreover, when orally administered in diabetic rats, the GLP-1 and DPP4 inhibitor loaded NPs showed a hypoglycemic effect as compared to the drug solution and empty NPs. Thus, this co-therapeutic multifunctional nanosystem exhibited high clinical potential as an oral nanocarrier for GLP-1 in combination with DPP4 inhibitor.

Finally, mucoadhesive and permeation enhancement of CSUn NPs were further enhanced by modifying the NPs' surface either by thiolation or by addition of CPP. As compared to CSUn NPs, L-cysteine and CPP modifications showed 17-fold and 12-fold enhancement in the insulin permeability across the intestinal cell monolayers, respectively. Furthermore, the involvement of several pathways in the uptake and transport of insulin across intestinal cell monolayers from different NPs were also observed. Electrostatic interactions between the NPs and the cell membrane were the most dominant mechanism, with the involvement of active transport as well. In addition, adsorptive endocytosis and clathrin-mediated endocytosis were also shown to be involved to some extent in the insulin

uptake and transport mechanisms. Overall, it was shown that further modifications of the CSUn NPs with either thiolation or CPP could significantly enhance the interactions with the intestinal cells and the intestinal permeability of insulin, thus displaying potential for improving oral insulin delivery systems.

In conclusion, in this dissertation, PSi, polymer and lipid-based systems with surface modifications using different mucoadhesive and permeation enhancing excipients and enteric coatings were developed. The potential of these nanosystems for oral insulin/GLP-1 delivery were assessed. Overall, the PSi-based platforms showed great potential for oral delivery of proteins and peptides for the DM therapy.

References

1. Craik DJ, Fairlie DP, Liras S, Price D, *The Future of peptide-based drugs.* Chem Biol Drug Des, 2013. **81**(1): p. 136-147.
2. Khafagy E-S, Morishita M, Onuki Y, Takayama K, *Current challenges in non-invasive insulin delivery systems: A comparative review.* Adv Drug Delivery Rev, 2007. **59**(15): p. 1521-1546.
3. Walsh G, *Biopharmaceutical benchmarks 2014.* Nat Biotechnol, 2014. **32**(10): p. 992-1000.
4. Herrero EP, Alonso MJ, Csaba N, *Polymer-based oral peptide nanomedicines.* Ther Deliv, 2012. **3**(5): p. 657-668.
5. Chen MC, Sonaje K, Chen KJ, Sung HW, *A review of the prospects for polymeric nanoparticle platforms in oral insulin delivery.* Biomaterials, 2011. **32**(36): p. 9826-9838.
6. Skyler JS, Cefalu WT, Kourides IA, Landschulz WH, Balagtas CC, Cheng SL, Gelfand RA, *Efficacy of inhaled human insulin in type 1 diabetes mellitus: A randomised proof-of-concept study.* Lancet, 2001. **357**(9253): p. 331-335.
7. Sonia TA, Sharma CP, *2 - Routes of administration of insulin*, in *Oral Delivery of Insulin*, TA Sonia and CP Sharma, Editors. 2014, Woodhead Publishing. p. 59-112.
8. Mukhopadhyay P, Mishra R, Rana D, Kundu PP, *Strategies for effective oral insulin delivery with modified chitosan nanoparticles: A review.* Prog Polym Sci, 2012. **37**(11): p. 1457-1475.
9. Steinert RE, Poller B, Castelli MC, Friedman K, Huber AR, Drewe J, Beglinger C, *Orally administered glucagon-like peptide-1 affects glucose homeostasis following an oral glucose tolerance test in healthy male subjects.* Clin Pharmacol Ther, 2009. **86**(6): p. 644-650.
10. Renukuntla J, Vadlapudi AD, Patel A, Boddu SHS, Mitra AK, *Approaches for enhancing oral bioavailability of peptides and proteins.* Int J Pharm, 2013. **447**(1-2): p. 75-93.
11. Salama NN, Eddington ND, Fasano A, *Tight junction modulation and its relationship to drug delivery.* Adv Drug Deliv Rev, 2006. **58**(1): p. 15-28.
12. Morishita M, Peppas NA, *Is the oral route possible for peptide and protein drug delivery.* Drug Discov Today, 2006. **11**(19-20): p. 905-910.
13. Park K, Kwon IC, Park K, *Oral protein delivery: Current status and future prospect.* React Funct Polym, 2011. **71**(3): p. 280-287.
14. Fonte P, Araujo F, Reis S, Sarmento B, *Oral insulin delivery: how far are we?* J Diabetes Sci Technol, 2013. **7**(2): p. 520-531.
15. Carino GP, Mathiowitz E, *Oral insulin delivery.* Adv Drug Deliv Rev, 1999. **35**(2-3): p. 249-257.
16. Shahbazi M-A, A. Santos H, *Improving oral absorption via drug-loaded nanocarriers: absorption mechanisms, intestinal models and rational fabrication.* Curr Drug Metab, 2013. **14**(1): p. 28-56.
17. Shan W, Zhu X, Liu M, Li L, Zhong J, Sun W, Zhang Z, Huang Y, *Overcoming the diffusion barrier of mucus and absorption barrier of epithelium by self-assembled nanoparticles for oral delivery of insulin.* ACS Nano, 2015. **9**(3): p. 2345-2356.
18. Araujo F, Shrestha N, Shahbazi MA, Liu D, Herranz-Blanco B, Makila EM, Salonen JJ, Hirvonen JT, Granja PL, Sarmento B, Santos HA, *Microfluidic assembly of a multifunctional tailorable composite system designed for site specific combined oral delivery of peptide drugs.* ACS Nano, 2015. **9**(8): p. 8291-8303.
19. Fonte P, Nogueira T, Gehm C, Ferreira D, Sarmento B, *Chitosan-coated solid lipid nanoparticles enhance the oral absorption of insulin.* Drug Delivery and Translational Research, 2011. **1**(4): p. 299-308.
20. Liu X, Liu C, Zhang W, Xie C, Wei G, Lu W, *Oligoarginine-modified biodegradable nanoparticles improve the intestinal absorption of insulin.* Int J Pharm, 2013. **448**(1): p. 159-167.
21. Liu D, Bimbo LM, Makila E, Villanova F, Kaasalainen M, Herranz-Blanco B, Caramella CM, Lehto VP, Salonen J, Herzig KH, Hirvonen J, Santos HA, *Co-delivery of a hydrophobic small molecule and a hydrophilic peptide by porous silicon nanoparticles.* J Control Release, 2013. **170**(2): p. 268-278.
22. Kovalainen M, Mönkäre J, Mäkilä E, Salonen J, Lehto V-P, Herzig K-H, Järvinen K, *Mesoporous silicon (PSi) for sustained peptide delivery: Effect of psi microparticle surface chemistry on peptide YY3-36 release.* Pharm Res, 2012. **29**(3): p. 837-846.
23. Kaasalainen M, Makila E, Riikonen J, Kovalainen M, Jarvinen K, Herzig KH, Lehto VP, Salonen J, *Effect of isotonic solutions and peptide adsorption on zeta potential of porous silicon nanoparticle drug delivery formulations.* Int J Pharm, 2012. **431**(1-2): p. 230-236.

24. Minardi S, Pandolfi L, Taraballi F, De Rosa E, Yazdi IK, Liu X, Ferrari M, Tasciotti E, *PLGA-mesoporous silicon microspheres for the in vivo controlled temporospatial delivery of proteins.* Acs Appl Mater Inter, 2015. **7**(30): p. 16364-16373.
25. Salonen J, Kaukonen AM, Hirvonen J, Lehto V-P, *Mesoporous silicon in drug delivery applications.* J Pharm Sci, 2008. **97**(2): p. 632-653.
26. Santos HA, Bimbo LM, Lehto VP, Airaksinen AJ, Salonen J, Hirvonen J, *Multifunctional porous silicon for therapeutic drug delivery and imaging.* Curr Drug Disc Technol, 2011. **8**(3): p. 228-249.
27. Savage DJ, Liu X, Curley SA, Ferrari M, Serda RE, *Porous silicon advances in drug delivery and immunotherapy.* Curr Opin Pharmacol, 2013. **13**(5): p. 834-841.
28. Sonaje K, Chuang EY, Lin KJ, Yen TC, Su FY, Tseng MT, Sung HW, *Opening of epithelial tight junctions and enhancement of paracellular permeation by chitosan: microscopic, ultrastructural, and computed-tomographic observations.* Mol Pharmaceutics, 2012. **9**(5): p. 1271-1279.
29. Baldrick P, *The safety of chitosan as a pharmaceutical excipient.* Regul Toxicol Pharm, 2010. **56**(3): p. 290-299.
30. Bernkop-Schnurch A, Hornof M, Guggi D, *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V.* Eur J Pharm Biopharm, 2004. **57**(1): p. 9-17.
31. Wang FH, Wang Y, Zhang X, Zhang WJ, Guo SR, Jin F, *Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery.* J Control Release, 2014. **174**: p. 126-136.
32. Kompella UB, Lee VHL, *Delivery systems for penetration enhancement of peptide and protein drugs: design considerations.* Adv Drug Delivery Rev, 2001. **46**(1–3): p. 211-245.
33. Eerikainen H, Watanabe W, Kauppinen EI, Ahonen PP, *Aerosol flow reactor method for synthesis of drug nanoparticles.* Eur J Pharm Biopharm, 2003. **55**(3): p. 357-360.
34. Raula J, Hanzlíková M, Rahikkala A, Hautala J, Kauppinen EI, Urtti A, Yliperttula M, *Gas-phase synthesis of solid state DNA nanoparticles stabilized by l-leucine.* Int J Pharm, 2013. **444**(1–2): p. 155-161.
35. Raula J, Rahikkala A, Halkola T, Pessi J, Peltonen L, Hirvonen J, Järvinen K, Laaksonen T, Kauppinen EI, *Coated particle assemblies for the concomitant pulmonary administration of budesonide and salbutamol sulphate.* Int J Pharm, 2013. **441**(1–2): p. 248-254.
36. Nykanen A, Rahikkala A, Hirvonen SP, Aseyev V, Tenhu H, Mezzenga R, Raula J, Kauppinen E, Ruokolainen J, *Thermally sensitive block copolymer particles prepared via aerosol flow reactor method: Morphological characterization and behavior in water.* Macromolecules, 2012. **45**(20): p. 8401-8411.
37. Eerikainen H, Kauppinen EI, *Preparation of polymeric nanoparticles containing corticosteroid by a novel aerosol flow reactor method.* Int J Pharm, 2003. **263**(1-2): p. 69-83.
38. Raula J, Eerikainen H, Kauppinen EI, *Influence of the solvent composition on the aerosol synthesis of pharmaceutical polymer nanoparticles.* Int J Pharm, 2004. **284**(1-2): p. 13-21.
39. Alberti KG, Zimmet PZ, *Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation.* Diabet Med, 1998. **15**(7): p. 539-553.
40. American Diabetes A, *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2013. **36 Suppl 1**: p. S67-74.
41. American Diabetes A, *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2010. **33 Suppl 1**: p. S62-9.
42. *International Diabetes Federation*. [cited 2015 Nov 29]; Available from: http://www.idf.org/.
43. Atkinson MA, Eisenbarth GS, Michels AW, *Type 1 diabetes.* Lancet, 2014. **383**(9911): p. 69-82.
44. Bluestone JA, Herold K, Eisenbarth G, *Genetics, pathogenesis and clinical interventions in type 1 diabetes.* Nature, 2010. **464**(7293): p. 1293-1300.
45. Alam U, Asghar O, Azmi S, Malik RA, *Chapter 15 - General aspects of diabetes mellitus*, in *Handb Clin Neurol*, WZ Douglas and AM Rayaz, Editors. 2014, Elsevier. p. 211-222.
46. Holman RR, Cull CA, Fox C, Turner RC, *United Kingdom Prospective Diabetes Study (UKPDS). 13: Relative efficacy of randomly allocated diet, sulphonylurea, insulin, or metformin in patients with newly diagnosed non-insulin dependent diabetes followed for three years.* BMJ, 1995. **310**(6972): p. 83-88.
47. Group CTDR, *Effect of intensive diabetes management on macrovascular events and risk factors in the Diabetes Control and Complications Trial.* Am J Cardiol, 1995. **75**(14): p. 894-903.

48. Thomas R, Home P, *Current insulin therapies for people with type 1 diabetes.* Prescriber, 2007. **18**(23-24): p. 25-41.
49. Fowler MJ, *Diabetes Treatment, Part 3: Insulin and Incretins.* Clin Diabetes, 2008. **26**(1): p. 35-39.
50. McCall AL, *Insulin therapy and hypoglycemia.* Endocrinol Metab Clin North Am, 2012. **41**(1): p. 57-87.
51. Andoh T, *Subchapter 19A - Insulin*, in *Handbook of Hormones*, YTA Tsutsui, Editor. 2016, Academic Press: San Diego. p. 157-160.
52. Curry DL, Bennett LL, Grodsky GM, *Dynamics of insulin secretion by the perfused rat pancreas.* Endocrinology, 1968. **83**(3): p. 572-584.
53. Seino S, Shibasaki T, Minami K, *Dynamics of insulin secretion and the clinical implications for obesity and diabetes.* J Clin Invest, 2011. **121**(6): p. 2118-2125.
54. McCrimmon RJ, Frier BM, *Hypoglycaemia, the most feared complication of insulin therapy.* Diabete Metab, 1994. **20**(6): p. 503-512.
55. Valla V, *Therapeutics of diabetes mellitus: focus on insulin analogues and insulin pumps.* Exp Diabetes Res, 2010. **2010**: p. 178372.
56. Krentz AJ, Bailey CJ, *Oral antidiabetic agents: current role in type 2 diabetes mellitus.* Drugs, 2005. **65**(3): p. 385-411.
57. Elrick H, Stimmler L, Hlad CJ, Jr., Arai Y, *Plasma insulin response to oral and intravenous glucose administration.* J Clin Endocrinol Metab, 1964. **24**: p. 1076-1082.
58. McIntyre N, Holdsworth CD, Turner DS, *New interpretation of oral glucose tolerance.* Lancet, 1964. **2**(7349): p. 20-21.
59. Baggio LL, Drucker DJ, *Biology of incretins: GLP-1 and GIP.* Gastroenterology, 2007. **132**(6): p. 2131-2157.
60. Dupre J, Ross SA, Watson D, Brown JC, *Stimulation of insulin secretion by gastric inhibitory polypeptide in man.* J Clin Endocrinol Metab, 1973. **37**(5): p. 826-828.
61. Lauritsen KB, Moody AJ, Christensen KC, Lindkaer Jensen S, *Gastric inhibitory polypeptide (GIP) and insulin release after small-bowel resection in man.* Scand J Gastroenterol, 1980. **15**(7): p. 833-840.
62. Todd JF, Bloom SR, *Incretins and other peptides in the treatment of diabetes.* Diabet Med, 2007. **24**(3): p. 223-232.
63. Kieffer TJ, Habener JF, *The glucagon-like peptides.* Endocr Rev, 1999. **20**(6): p. 876-913.
64. Holst J, Ørskov C, Nielsen OV, Schwartz T, *Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut.* FEBS Lett, 1987. **211**(2): p. 169-174.
65. Verspohl EJ, *Novel therapeutics for type 2 diabetes: incretin hormone mimetics (glucagon-like peptide-1 receptor agonists) and dipeptidyl peptidase-4 inhibitors.* Pharmacol Ther, 2009. **124**(1): p. 113-138.
66. Holst JJ, Gromada J, *Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans.* Am J Physiol Endocrinol Metab, 2004. **287**(2): p. E199-206.
67. Nauck M, Stockmann F, Ebert R, Creutzfeldt W, *Reduced incretin effect in type 2 (non-insulin-dependent) diabetes.* Diabetologia, 1986. **29**(1): p. 46-52.
68. Kazafeos K, *Incretin effect: GLP-1, GIP, DPP4.* Diabetes Res Clin Pract, 2011. **93 Suppl 1**: p. S32-36.
69. Nauck MA, Heimesaat MM, Orskov C, Holst JJ, Ebert R, Creutzfeldt W, *Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus.* J Clin Invest, 1993. **91**(1): p. 301-307.
70. Qualmann C, Nauck MA, Holst JJ, Orskov C, Creutzfeldt W, *Insulinotropic actions of intravenous glucagon-like peptide-1 (GLP-1) [7-36 amide] in the fasting state in healthy subjects.* Acta Diabetol, 1995. **32**(1): p. 13-16.
71. Nauck MA, Heimesaat MM, Behle K, Holst JJ, Nauck MS, Ritzel R, Hufner M, Schmiegel WH, *Effects of glucagon-like peptide 1 on counterregulatory hormone responses, cognitive functions, and insulin secretion during hyperinsulinemic, stepped hypoglycemic clamp experiments in healthy volunteers.* J Clin Endocrinol Metab, 2002. **87**(3): p. 1239-1246.
72. Drucker DJ, *The biology of incretin hormones.* Cell Metab, 2006. **3**(3): p. 153-165.
73. Mentlein R, *Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides.* Regul Pept, 1999. **85**(1): p. 9-24.

74. Mentlein R, Gallwitz B, Schmidt WE, *Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum.* Eur J Biochem, 1993. **214**(3): p. 829-835.
75. Hansen L, Deacon CF, Orskov C, Holst JJ, *Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine.* Endocrinology, 1999. **140**(11): p. 5356-5363.
76. Ahren B, *DPP-4 inhibitors.* Best Pract Res Clin Endocrinol Metab, 2007. **21**(4): p. 517-533.
77. Ahren B, Gomis R, Standl E, Mills D, Schweizer A, *Twelve- and 52-week efficacy of the dipeptidyl peptidase IV inhibitor LAF237 in metformin-treated patients with type 2 diabetes.* Diabetes Care, 2004. **27**(12): p. 2874-2880.
78. Ahren B, Simonsson E, Larsson H, Landin-Olsson M, Torgeirsson H, Jansson PA, Sandqvist M, Bavenholm P, Efendic S, Eriksson JW, Dickinson S, Holmes D, *Inhibition of dipeptidyl peptidase IV improves metabolic control over a 4-week study period in type 2 diabetes.* Diabetes Care, 2002. **25**(5): p. 869-875.
79. Ahren B, Pacini G, Foley JE, Schweizer A, *Improved meal-related beta-cell function and insulin sensitivity by the dipeptidyl peptidase-IV inhibitor vildagliptin in metformin-treated patients with type 2 diabetes over 1 year.* Diabetes Care, 2005. **28**(8): p. 1936-1940.
80. Lankas GR, Leiting B, Roy RS, Eiermann GJ, Beconi MG, Biftu T, Chan CC, Edmondson S, Feeney WP, He H, Ippolito DE, Kim D, Lyons KA, Ok HO, Patel RA, Petrov AN, Pryor KA, Qian X, Reigle L, Woods A, Wu JK, Zaller D, Zhang X, Zhu L, Weber AE, Thornberry NA, *Dipeptidyl peptidase IV inhibition for the treatment of type 2 diabetes: potential importance of selectivity over dipeptidyl peptidases 8 and 9.* Diabetes, 2005. **54**(10): p. 2988-2994.
81. Hughes TE, Mone MD, Russell ME, Weldon SC, Villhauer EB, *NVP-DPP728 (1-[[[2-[(5-cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(S)- pyrrolidine), a slow-binding inhibitor of dipeptidyl peptidase IV.* Biochemistry, 1999. **38**(36): p. 11597-11603.
82. Villhauer EB, Brinkman JA, Naderi GB, Dunning BE, Mangold BL, Mone MD, Russell ME, Weldon SC, Hughes TE, *1-[2-[(5-Cyanopyridin-2-yl)amino]ethylamino]acetyl-2-(S)-pyrrolidinecarbonitrile : A potent, selective, and orally bioavailable dipeptidyl peptidase IV inhibitor with antihyperglycemic properties.* J Med Chem, 2002. **45**(12): p. 2362-2365.
83. Reimer MK, Holst JJ, Ahren B, *Long-term inhibition of dipeptidyl peptidase IV improves glucose tolerance and preserves islet function in mice.* Eur J Endocrinol, 2002. **146**(5): p. 717-727.
84. Balkan B, Kwasnik L, Miserendino R, Holst JJ, Li X, *Inhibition of dipeptidyl peptidase IV with NVP-DPP728 increases plasma GLP-1 (7-36 amide) concentrations and improves oral glucose tolerance in obese Zucker rats.* Diabetologia, 1999. **42**(11): p. 1324-1331.
85. Al-Tabakha MM, Arida AI, *Recent challenges in insulin delivery systems: a review.* Indian J Pharm Sci, 2008. **70**(3): p. 278-286.
86. Kwon K-C, Verma D, Singh ND, Herzog R, Daniell H, *Oral delivery of human biopharmaceuticals, autoantigens and vaccine antigens bioencapsulated in plant cells.* Adv Drug Delivery Rev, 2013. **65**(6): p. 782-799.
87. Agarwal V, Khan MA, *Current status of the oral delivery of insulin.* Pharm Technol North Am, 2001. **25**(10): p. 76-90.
88. Fonte P, Araújo F, Silva C, Pereira C, Reis S, Santos HA, Sarmento B, *Polymer-based nanoparticles for oral insulin delivery: Revisited approaches.* Biotechnol Adv, 2015. **33**(6, Part 3): p. 1342-1354.
89. *Diabetology*. [cited 2015 Nov 24]; Available from: http://www.diabetology.co.uk/.
90. *Oramed Pharamceuticals*. [cited 2015 Nov 24]; Available from: http://www.oramed.com/.
91. *Novo Nordisk*. [cited 2016 Jan 8]; Available from: http://www.novonordisk.com/.
92. *Diasome Pharamceuticals*. [cited 2016 Jan 2]; Available from: http://www.diasome.com/.
93. Ensign LM, Cone R, Hanes J, *Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers.* Adv Drug Deliv Rev, 2012. **64**(6): p. 557-570.
94. Balcerzak SP, Lane WC, Bullard JW, *Surface structure of intestinal epithelium.* Gastroenterology, 1970. **58**(1): p. 49-55.
95. Cheng H, *Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. II. Mucous cells.* Am J Anat, 1974. **141**(4): p. 481-501.
96. Kiyono H, Fukuyama S, *NALT- versus Peyer's-patch-mediated mucosal immunity.* Nat Rev Immunol, 2004. **4**(9): p. 699-710.

97. Hayakawa E, Lee VH, *Aminopeptidase activity in the jejunal and ileal Peyer's patches of the albino rabbit.* Pharm Res, 1992. **9**(4): p. 535-540.
98. Maroni A, Zema L, Del Curto MD, Foppoli A, Gazzaniga A, *Oral colon delivery of insulin with the aid of functional adjuvants.* Adv Drug Delivery Rev, 2012. **64**(6): p. 540-556.
99. Nusrat A, Turner JR, Madara JL, *Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells.* Am J Physiol Gastrointest Liver Physiol, 2000. **279**(5): p. G851-857.
100. Chen M-C, Sonaje K, Chen K-J, Sung H-W, *A review of the prospects for polymeric nanoparticle platforms in oral insulin delivery.* Biomaterials, 2011. **32**(36): p. 9826-9838.
101. Anderson JM, Van Itallie CM, *Tight junctions and the molecular basis for regulation of paracellular permeability.* Am J Physiol, 1995. **269**(4 Pt 1): p. G467-475.
102. Yun Y, Cho YW, Park K, *Nanoparticles for oral delivery: Targeted nanoparticles with peptidic ligands for oral protein delivery.* Adv Drug Delivery Rev, 2013. **65**(6): p. 822-832.
103. Thornton DJ, Sheehan JK, *From mucins to mucus: toward a more coherent understanding of this essential barrier.* Proc Am Thorac Soc, 2004. **1**(1): p. 54-61.
104. Cone RA, *Barrier properties of mucus.* Adv Drug Delivery Rev, 2009. **61**(2): p. 75-85.
105. Sosnik A, das Neves J, Sarmento B, *Mucoadhesive polymers in the design of nano-drug delivery systems for administration by non-parenteral routes: A review.* Prog Polym Sci, 2014. **39**(12): p. 2030-2075.
106. Lai SK, Wang YY, Wirtz D, Hanes J, *Micro- and macrorheology of mucus.* Adv Drug Deliv Rev, 2009. **61**(2): p. 86-100.
107. Clamp JR, Creeth JM, *Some non-mucin components of mucus and their possible biological roles.* Ciba Foundation Symposia, 1984. **109**: p. 121-136.
108. Sheehan JK, Oates K, Carlstedt I, *Electron-microscopy of cervical, gastric and bronchial mucus glycoproteins.* Biochem J, 1986. **239**(1): p. 147-153.
109. Lai SK, Wang Y-Y, Hanes J, *Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues.* Adv Drug Delivery Rev, 2009. **61**(2): p. 158-171.
110. Lehr C-M, Poelma FGJ, Junginger HE, Tukker JJ, *An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop.* Int J Pharm, 1991. **70**(3): p. 235-240.
111. Woodley JF, *Enzymatic barriers for GI peptide and protein delivery.* Crit Rev Ther Drug Carrier Syst, 1994. **11**(2-3): p. 61-95.
112. Langguth P, Bohner V, Heizmann J, Merkle HP, Wolffram S, Amidon GL, Yamashita S, *The challenge of proteolytic enzymes in intestinal peptide delivery.* J Control Release, 1997. **46**(1-2): p. 39-57.
113. Sugano K, Kansy M, Artursson P, Avdeef A, Bendels S, Di L, Ecker GF, Faller B, Fischer H, Gerebtzoff G, Lennernaes H, Senner F, *Coexistence of passive and carrier-mediated processes in drug transport.* Nat Rev Drug Discov, 2010. **9**(8): p. 597-614.
114. Pawar VK, Meher JG, Singh Y, Chaurasia M, Surendar Reddy B, Chourasia MK, *Targeting of gastrointestinal tract for amended delivery of protein/peptide therapeutics: strategies and industrial perspectives.* J Control Release, 2014. **196**: p. 168-183.
115. Salamat-Miller N, Johnston TP, *Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium.* Int J Pharm, 2005. **294**(1-2): p. 201-216.
116. Van Itallie CM, Holmes J, Bridges A, Gookin JL, Coccaro MR, Proctor W, Colegio OR, Anderson JM, *The density of small tight junction pores varies among cell types and is increased by expression of claudin-2.* J Cell Sci, 2008. **121**(Pt 3): p. 298-305.
117. Tomita M, Shiga M, Hayashi M, Awazu S, *Enhancement of colonic drug absorption by the paracellular permeation route.* Pharm Res, 1988. **5**(6): p. 341-346.
118. Shakweh M, Ponchel G, Fattal E, *Particle uptake by Peyer's patches: a pathway for drug and vaccine delivery.* Expert Opin Drug Deliv, 2004. **1**(1): p. 141-163.
119. Behrens I, Pena AI, Alonso MJ, Kissel T, *Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport.* Pharm Res, 2002. **19**(8): p. 1185-1193.
120. Kadiyala I, Loo Y, Roy K, Rice J, Leong KW, *Transport of chitosan–DNA nanoparticles in human intestinal M-cell model versus normal intestinal enterocytes.* Eur J Pharm Sci, 2010. **39**(1–3): p. 103-109.

121. des Rieux A, Fievez V, Garinot M, Schneider Y-J, Préat V, *Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach.* J Control Release, 2006. **116**(1): p. 1-27.
122. Conner SD, Schmid SL, *Regulated portals of entry into the cell.* Nature, 2003. **422**(6927): p. 37-44.
123. Goldberg M, Gomez-Orellana I, *Challenges for the oral delivery of macromolecules.* Nat Rev Drug Discov, 2003. **2**(4): p. 289-295.
124. R TB, Jeffrey A, Siahaan TJ, Gangwar S, Pauletti GM, *Improvement of oral peptide bioavailability: Peptidomimetics and prodrug strategies.* Adv Drug Deliv Rev, 1997. **27**(2-3): p. 235-256.
125. Niu M, Lu Y, Hovgaard L, Guan P, Tan Y, Lian R, Qi J, Wu W, *Hypoglycemic activity and oral bioavailability of insulin-loaded liposomes containing bile salts in rats: The effect of cholate type, particle size and administered dose.* Eur J Pharm Biopharm, 2012. **81**(2): p. 265-272.
126. Yin L, Ding J, He C, Cui L, Tang C, Yin C, *Drug permeability and mucoadhesion properties of thiolated trimethyl chitosan nanoparticles in oral insulin delivery.* Biomaterials, 2009. **30**(29): p. 5691-5700.
127. Kamm W, Jonczyk A, Jung T, Luckenbach G, Raddatz P, Kissel T, *Evaluation of absorption enhancement for a potent cyclopeptidic αvβ3-antagonist in a human intestinal cell line (Caco-2).* Eur J Pharm Sci, 2000. **10**(3): p. 205-214.
128. Su F-Y, Lin K-J, Sonaje K, Wey S-P, Yen T-C, Ho Y-C, Panda N, Chuang E-Y, Maiti B, Sung H-W, *Protease inhibition and absorption enhancement by functional nanoparticles for effective oral insulin delivery.* Biomaterials, 2012. **33**(9): p. 2801-2811.
129. Salama NN, Fasano A, Lu R, Eddington ND, *Effect of the biologically active fragment of zonula occludens toxin, ΔG, on the intestinal paracellular transport and oral absorption of mannitol.* Int J Pharm, 2003. **251**(1–2): p. 113-121.
130. Sood A, Panchagnula R, *Peroral route: an opportunity for protein and peptide drug delivery.* Chem Rev, 2001. **101**(11): p. 3275-3303.
131. Hearnden V, Sankar V, Hull K, Juras DV, Greenberg M, Kerr AR, Lockhart PB, Patton LL, Porter S, Thornhill MH, *New developments and opportunities in oral mucosal drug delivery for local and systemic disease.* Adv Drug Deliv Rev, 2012. **64**(1): p. 16-28.
132. Whitehead K, Karr N, Mitragotri S, *Safe and effective permeation enhancers for oral drug delivery.* Pharm Res, 2008. **25**(8): p. 1782-1788.
133. Friedman DI, Amidon GL, *Oral absorption of peptides: influence of pH and inhibitors on the intestinal hydrolysis of leu-enkephalin and analogues.* Pharm Res, 1991. **8**(1): p. 93-96.
134. Marschutz MK, Bernkop-Schnurch A, *Oral peptide drug delivery: polymer-inhibitor conjugates protecting insulin from enzymatic degradation in vitro.* Biomaterials, 2000. **21**(14): p. 1499-1507.
135. Yamamoto A, Taniguchi T, Rikyuu K, Tsuji T, Fujita T, Murakami M, Muranishi S, *Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats.* Pharm Res, 1994. **11**(10): p. 1496-1500.
136. Rekha MR, Sharma CP, *Oral delivery of therapeutic protein/peptide for diabetes – Future perspectives.* Int J Pharm, 2013. **440**(1): p. 48-62.
137. Bernkop-Schnurch A, Weithaler A, Albrecht K, Greimel A, *Thiomers: preparation and in vitro evaluation of a mucoadhesive nanoparticulate drug delivery system.* Int J Pharm, 2006. **317**(1): p. 76-81.
138. Sung HW, Sonaje K, Liao ZX, Hsu LW, Chuang EY, *pH-responsive nanoparticles shelled with chitosan for oral delivery of insulin: from mechanism to therapeutic applications.* Acc Chem Res, 2012. **45**(4): p. 619-629.
139. Khutoryanskiy VV, *Advances in mucoadhesion and mucoadhesive polymers.* Macromol Biosci, 2011. **11**(6): p. 748-764.
140. Smart JD, *The basics and underlying mechanisms of mucoadhesion.* Adv Drug Deliv Rev, 2005. **57**(11): p. 1556-1568.
141. Peppas NA, Sahlin JJ, *Hydrogels as mucoadhesive and bioadhesive materials: A review.* Biomaterials, 1996. **17**(16): p. 1553-1561.
142. Sarciaux JM, Acar L, Sado PA, *Using microemulsion formulations for oral drug delivery of therapeutic peptides.* Int J Pharm, 1995. **120**(2): p. 127-136.
143. Fievez V, Plapied L, des Rieux A, Pourcelle V, Freichels H, Wascotte V, Vanderhaeghen ML, Jerome C, Vanderplasschen A, Marchand-Brynaert J, Schneider YJ, Preat V, *Targeting nanoparticles to M cells with non-peptidic ligands for oral vaccination.* Eur J Pharm Biopharm, 2009. **73**(1): p. 16-24.

144. Araujo F, Shrestha N, Granja PL, Hirvonen J, Santos HA, Sarmento B, *Safety and toxicity concerns of orally delivered nanoparticles as drug carriers.* Expert Opin Drug Metab Toxicol, 2014: p. 1-13.
145. Bakhru SH, Furtado S, Morello AP, Mathiowitz E, *Oral delivery of proteins by biodegradable nanoparticles.* Adv Drug Delivery Rev, 2013. **65**(6): p. 811-821.
146. Desai MP, Labhasetwar V, Walter E, Levy RJ, Amidon GL, *The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent.* Pharm Res, 1997. **14**(11): p. 1568-1573.
147. Uhlir A, *Electrolytic shaping of germanium and silicon.* Bell System Technical Journal, 1956. **35**(2): p. 333-347.
148. Watanabe Y, Sakai T, *Application of a thick anode film to semiconductor devices.* Review of The Electrical Communications Laboratories, 1971. **19**(7-8): p. 899.
149. Canham LT, *Bioactive silicon structure fabrication through nanoetching techniques.* Adv Mater, 1995. **7**(12): p. 1033-1037.
150. Canham L, *Biomedical applications of porous silicon.* Properties of porous silicon, 1997. **18**: p. 12-22.
151. Santos HA, Makila E, Airaksinen AJ, Bimbo LM, Hirvonen J, *Porous silicon nanoparticles for nanomedicine: Preparation and biomedical applications.* Nanomedicine (Lond), 2014. **9**(4): p. 535-554.
152. Haidary SM, Corcoles EP, Ali NK, *Nanoporous silicon as drug delivery systems for cancer therapies.* Journal of Nanomaterials, 2012. **2012**(2012): p. 1-15.
153. Shahbazi MA, Herranz B, Santos HA, *Nanostructured porous Si-based nanoparticles for targeted drug delivery.* Biomatter, 2012. **2**(4): p. 296-312.
154. Makila E, Ferreira MP, Kivela H, Niemi SM, Correia A, Shahbazi MA, Kauppila J, Hirvonen J, Santos HA, Salonen J, *Confinement effects on drugs in thermally hydrocarbonized porous silicon.* Langmuir, 2014. **30**(8): p. 2196-205.
155. Anglin EJ, Cheng L, Freeman WR, Sailor MJ, *Porous silicon in drug delivery devices and materials.* Adv Drug Deliv Rev, 2008. **60**(11): p. 1266-1277.
156. Shahbazi MA, Almeida PV, Makila EM, Kaasalainen MH, Salonen JJ, Hirvonen JT, Santos HA, *Augmented cellular trafficking and endosomal escape of porous silicon nanoparticles via zwitterionic bilayer polymer surface engineering.* Biomaterials, 2014. **35**(26): p. 7488-7500.
157. Wang CF, Makila EM, Kaasalainen MH, Liu DF, Sarparanta MP, Airaksinen AJ, Salonen JJ, Hirvonen JT, Santos HA, *Copper-free azide-alkyne cycloaddition of targeting peptides to porous silicon nanoparticles for intracellular drug uptake.* Biomaterials, 2014. **35**(4): p. 1257-1266.
158. Bimbo LM, Makila E, Raula J, Laaksonen T, Laaksonen P, Strommer K, Kauppinen EI, Salonen J, Linder MB, Hirvonen J, Santos HA, *Functional hydrophobin-coating of thermally hydrocarbonized porous silicon microparticles.* Biomaterials, 2011. **32**(34): p. 9089-9099.
159. Salonen J, Lehto V-P, *Fabrication and chemical surface modification of mesoporous silicon for biomedical applications.* Chem Eng J, 2008. **137**(1): p. 162-172.
160. Jalkanen T, Maattanen A, Makila E, Tuura J, Kaasalainen M, Lehto VP, Ihalainen P, Peltonen J, Salonen J, *Fabrication of porous silicon based humidity sensing elements on paper.* J Sensors, 2015. **2015**(2015): p. 1-10.
161. Andersen O, Frello T, Veje E, *Photoinduced synthesis of porous silicon without anodization.* J Appl Phys, 1995. **78**(10): p. 6189-6192.
162. Fathauer R, George T, Ksendzov A, Vasquez R, *Visible luminescence from silicon wafers subjected to stain etches.* Appl Phys Lett, 1992. **60**(8): p. 995-997.
163. Riikonen J, Salomäki M, van Wonderen J, Kemell M, Xu W, Korhonen O, Ritala M, MacMillan F, Salonen J, Lehto V-P, *Surface Chemistry, Reactivity, and Pore Structure of Porous Silicon Oxidized by Various Methods.* Langmuir, 2012. **28**(28): p. 10573-10583.
164. Jarvis KL, Barnes TJ, Prestidge CA, *Surface chemistry of porous silicon and implications for drug encapsulation and delivery applications.* Adv Colloid Interface Sci, 2012. **175**: p. 25-38.
165. Salonen J, Björkqvist M, Laine E, Niinistö L, *Stabilization of porous silicon surface by thermal decomposition of acetylene.* Appl Surf Sci, 2004. **225**(1): p. 389-394.
166. Jalkanen T, Makila E, Sakka T, Salonen J, Ogata YH, *Thermally promoted addition of undecylenic acid on thermally hydrocarbonized porous silicon optical reflectors.* Nanoscale Res Lett, 2012. **7**(1): p. 311-317.

167. Bimbo LM, Sarparanta M, Santos HA, Airaksinen AJ, Makila E, Laaksonen T, Peltonen L, Lehto VP, Hirvonen J, Salonen J, *Biocompatibility of thermally hydrocarbonized porous silicon nanoparticles and their biodistribution in rats.* ACS Nano, 2010. **4**(6): p. 3023-3032.
168. Santos HA, Riikonen J, Salonen J, Makila E, Heikkila T, Laaksonen T, Peltonen L, Lehto VP, Hirvonen J, *In vitro cytotoxicity of porous silicon microparticles: effect of the particle concentration, surface chemistry and size.* Acta Biomater, 2010. **6**(7): p. 2721-2731.
169. Sarparanta M, Bimbo LM, Rytkonen J, Makila E, Laaksonen TJ, Laaksonen P, Nyman M, Salonen J, Linder MB, Hirvonen J, Santos HA, Airaksinen AJ, *Intravenous delivery of hydrophobin-functionalized porous silicon nanoparticles: Stability, plasma protein adsorption and biodistribution.* Mol Pharm, 2012. **9**(3): p. 654-663.
170. Shahbazi MA, Fernandez TD, Makila EM, Le Guevel X, Mayorga C, Kaasalainen MH, Salonen JJ, Hirvonen JT, Santos HA, *Surface chemistry dependent immunostimulative potential of porous silicon nanoplatforms.* Biomaterials, 2014. **35**(33): p. 9224-9235.
171. Shahbazi MA, Hamidi M, Makila EM, Zhang H, Almeida PV, Kaasalainen M, Salonen JJ, Hirvonen JT, Santos HA, *The mechanisms of surface chemistry effects of mesoporous silicon nanoparticles on immunotoxicity and biocompatibility.* Biomaterials, 2013. **34**(31): p. 7776-7789.
172. Tolli MA, Ferreira MP, Kinnunen SM, Rysa J, Makila EM, Szabo Z, Serpi RE, Ohukainen PJ, Valimaki MJ, Correia AM, Salonen JJ, Hirvonen JT, Ruskoaho HJ, Santos HA, *In vivo biocompatibility of porous silicon biomaterials for drug delivery to the heart.* Biomaterials, 2014. **35**(29): p. 8394-8405.
173. Bimbo LM, Makila E, Laaksonen T, Lehto VP, Salonen J, Hirvonen J, Santos HA, *Drug permeation across intestinal epithelial cells using porous silicon nanoparticles.* Biomaterials, 2011. **32**(10): p. 2625-2633.
174. Korhonen E, Rönkkö S, Hillebrand S, Riikonen J, Xu W, Järvinen K, Lehto V-P, Kauppinen A, *Cytotoxicity assessment of porous silicon microparticles for ocular drug delivery.* Eur J Pharm Biopharm, 2016. **100**: p. 1-8.
175. Salonen J, Laitinen L, Kaukonen AM, Tuura J, Björkqvist M, Heikkilä T, Vähä-Heikkilä K, Hirvonen J, Lehto VP, *Mesoporous silicon microparticles for oral drug delivery: Loading and release of five model drugs.* J Control Release, 2005. **108**(2–3): p. 362-374.
176. Tahvanainen M, Rotko T, Makila E, Santos HA, Neves D, Laaksonen T, Kallonen A, Hamalainen K, Peura M, Serimaa R, Salonen J, Hirvonen J, Peltonen L, *Tablet preformulations of indomethacin-loaded mesoporous silicon microparticles.* Int J Pharm, 2012. **422**(1-2): p. 125-131.
177. Wang F, Hui H, Barnes TJ, Barnett C, Prestidge CA, *Oxidized mesoporous silicon microparticles for improved oral delivery of poorly soluble drugs.* Mol Pharm, 2010. **7**(1): p. 227-236.
178. Alba-Simionesco C, Coasne B, Dosseh G, Dudziak G, Gubbins KE, Radhakrishnan R, Sliwinska-Bartkowiak M, *Effects of confinement on freezing and melting.* J Phys Condens Matter, 2006. **18**(6): p. R15-68.
179. Correia A, Shahbazi MA, Makila E, Almeida S, Salonen J, Hirvonen J, Santos HA, *Cyclodextrin-modified porous silicon nanoparticles for efficient sustained drug delivery and proliferation inhibition of breast cancer cells.* ACS Appl Mater Interfaces, 2015. **7**(41): p. 23197-23204.
180. Riikonen J, Correia A, Kovalainen M, Näkki S, Lehtonen M, Leppänen J, Rantanen J, Xu W, Araújo F, Hirvonen J, Järvinen K, Santos HA, Lehto V-P, *Systematic in vitro and in vivo study on porous silicon to improve the oral bioavailability of celecoxib.* Biomaterials, 2015. **52**: p. 44-55.
181. Wang CF, Makila EM, Kaasalainen MH, Hagstrom MV, Salonen JJ, Hirvonen JT, Santos HA, *Dual-drug delivery by porous silicon nanoparticles for improved cellular uptake, sustained release, and combination therapy.* Acta Biomaterialia, 2015. **16**: p. 206-214.
182. Kinnari P, Makila E, Heikkila T, Salonen J, Hirvonen J, Santos HA, *Comparison of mesoporous silicon and non-ordered mesoporous silica materials as drug carriers for itraconazole.* Int J Pharm, 2011. **414**(1-2): p. 148-156.
183. Vale N, Makila E, Salonen J, Gomes P, Hirvonen J, Santos HA, *New times, new trends for ethionamide: In vitro evaluation of drug-loaded thermally carbonized porous silicon microparticles.* Eur J Pharm Biopharm, 2012. **81**(2): p. 314-23.
184. Herranz-Blanco B, Arriaga LR, Makila E, Correia A, Shrestha N, Mirza S, Weitz DA, Salonen J, Hirvonen J, Santos HA, *Microfluidic assembly of multistage porous silicon-lipid vesicles for controlled drug release.* Lab Chip, 2014. **14**(6): p. 1083-1086.

185. Kong F, Zhang X, Zhang HB, Qu XM, Chen D, Servos M, Makila E, Salonen J, Santos HA, Hai MT, Weitz DA, *Inhibition of Multidrug Resistance of Cancer Cells by Co-Delivery of DNA Nanostructures and Drugs Using Porous Silicon Nanoparticles@Giant Liposomes.* Adv Funct Mater, 2015. **25**(22): p. 3330-3340.
186. Sarparanta MP, Bimbo LM, Makila EM, Salonen JJ, Laaksonen PH, Helariutta AM, Linder MB, Hirvonen JT, Laaksonen TJ, Santos HA, Airaksinen AJ, *The mucoadhesive and gastroretentive properties of hydrophobin-coated porous silicon nanoparticle oral drug delivery systems.* Biomaterials, 2012. **33**(11): p. 3353-3362.
187. De Angelis F, Pujia A, Falcone C, Iaccino E, Palmieri C, Liberale C, Mecarini F, Candeloro P, Luberto L, de Laurentiis A, Das G, Scala G, Di Fabrizio E, *Water soluble nanoporous nanoparticle for in vivo targeted drug delivery and controlled release in B cells tumor context.* Nanoscale, 2010. **2**(10): p. 2230-2236.
188. Wan Y, Apostolou S, Dronov R, Kuss B, Voelcker NH, *Cancer-targeting siRNA delivery from porous silicon nanoparticles.* Nanomedicine (Lond), 2014. **9**(15): p. 2309-2321.
189. Zhang M, Xu R, Xia X, Yang Y, Gu J, Qin G, Liu X, Ferrari M, Shen H, *Polycation-functionalized nanoporous silicon particles for gene silencing on breast cancer cells.* Biomaterials, 2014. **35**(1): p. 423-431.
190. Gaur G, Koktysh DS, Weiss SM, *Immobilization of quantum dots in nanostructured porous silicon films: Characterizations and signal amplification for dual-mode optical biosensing.* Adv Funct Mater, 2013. **23**(29): p. 3604-3614.
191. Tasciotti E, Liu X, Bhavane R, Plant K, Leonard AD, Price BK, Cheng MM-C, Decuzzi P, Tour JM, Robertson F, Ferrari M, *Mesoporous silicon particles as a multistage delivery system for imaging and therapeutic applications.* Nat Nano, 2008. **3**(3): p. 151-157.
192. Shen H, You J, Zhang G, Ziemys A, Li Q, Bai L, Deng X, Erm DR, Liu X, Li C, Ferrari M, *Cooperative, nanoparticle-enabled thermal therapy of breast cancer.* Adv Healthc Mater, 2012. **1**(1): p. 84-89.
193. Gu L, Park JH, Duong KH, Ruoslahti E, Sailor MJ, *Magnetic luminescent porous silicon microparticles for localized delivery of molecular drug payloads.* Small, 2010. **6**(22): p. 2546-2552.
194. Foraker AB, Walczak RJ, Cohen MH, Boiarski TA, Grove CF, Swaan PW, *Microfabricated porous silicon particles enhance paracellular delivery of insulin across intestinal Caco-2 cell monolayers.* Pharm Res, 2003. **20**(1): p. 110-116.
195. Liu D, Zhang H, Makila E, Fan J, Herranz-Blanco B, Wang CF, Rosa R, Ribeiro AJ, Salonen J, Hirvonen J, Santos HA, *Microfluidic assisted one-step fabrication of porous silicon@acetalated dextran nanocomposites for precisely controlled combination chemotherapy.* Biomaterials, 2015. **39**: p. 249-259.
196. Huotari A, Xu WJ, Monkare J, Kovalainen M, Herzig KH, Lehto VP, Jarvinen K, *Effect of surface chemistry of porous silicon microparticles on glucagon-like peptide-1 (GLP-1) loading, release and biological activity.* Int J Pharm, 2013. **454**(1): p. 67-73.
197. Kilpeläinen M, Mönkäre J, Vlasova MA, Riikonen J, Lehto V-P, Salonen J, Järvinen K, Herzig K-H, *Nanostructured porous silicon microparticles enable sustained peptide (Melanotan II) delivery.* Eur J Pharm Biopharm, 2011. **77**(1): p. 20-25.
198. Kilpeläinen M, Riikonen J, Vlasova MA, Huotari A, Lehto VP, Salonen J, Herzig KH, Järvinen K, *In vivo delivery of a peptide, ghrelin antagonist, with mesoporous silicon microparticles.* J Control Release, 2009. **137**(2): p. 166-170.
199. Kaasalainen M, Rytkonen J, Makila E, Narvanen A, Salonen J, *Electrostatic interaction on loading of therapeutic peptide GLP-1 into porous silicon nanoparticles.* Langmuir, 2015. **31**(5): p. 1722-1729.
200. Jarvis KL, Barnes TJ, Prestidge CA, *Thermal oxidation for controlling protein interactions with porous silicon.* Langmuir, 2010. **26**(17): p. 14316-14322.
201. Andrew JS, Anglin EJ, Wu EC, Chen MY, Cheng L, Freeman WR, Sailor MJ, *Sustained release of a monoclonal antibody from electrochemically prepared mesoporous silicon oxide.* Adv Funct Mater, 2010. **20**(23): p. 4168-4174.
202. Karlsson LM, Tengvall P, Lundström I, Arwin H, *Penetration and loading of human serum albumin in porous silicon layers with different pore sizes and thicknesses.* J Colloid Interface Sci, 2003. **266**(1): p. 40-47.

203. Prestidge CA, Barnes TJ, Mierczynska-Vasilevl A, Kempson I, Peddiel F, Barnett C, *Peptide and protein loading into porous silicon wafers.* Phys Status Solidi A, 2008. **205**(2): p. 311-315.
204. Pastor EL, Reguera-Nuñez E, Matveeva E, Garcia-Fuentes M, *Pore size is a critical parameter for obtaining sustained protein release from electrochemically synthesized mesoporous silicon microparticles.* PeerJ, 2015. **3**: p. e1277.
205. Pinto Reis C, Neufeld RJ, Ribeiro AJ, Veiga F, *Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles.* Nanomedicine, 2006. **2**(1): p. 8-21.
206. Sosnik A, Carcaboso AM, *Nanomedicines in the future of pediatric therapy.* Adv Drug Delivery Rev, 2014. **73**: p. 140-161.
207. Gan Q, Wang T, *Chitosan nanoparticle as protein delivery carrier—Systematic examination of fabrication conditions for efficient loading and release.* Colloids Surf B, 2007. **59**(1): p. 24-34.
208. Makhlof A, Tozuka Y, Takeuchi H, *Design and evaluation of novel pH-sensitive chitosan nanoparticles for oral insulin delivery.* Eur J Pharm Sci, 2011. **42**(5): p. 445-451.
209. Chalasani KB, Russell-Jones GJ, Yandrapu SK, Diwan PV, Jain SK, *A novel vitamin B12-nanosphere conjugate carrier system for peroral delivery of insulin.* J Control Release, 2007. **117**(3): p. 421-429.
210. Sarmento B, Ribeiro AJ, Veiga F, Ferreira DC, Neufeld RJ, *Insulin-loaded nanoparticles are prepared by alginate ionotropic pre-gelation followed by chitosan polyelectrolyte complexation.* J Nanosci Nanotechnol, 2007. **7**(8): p. 2833-2841.
211. Lin Y-H, Mi F-L, Chen C-T, Chang W-C, Peng S-F, Liang H-F, Sung H-W, *Preparation and characterization of nanoparticles shelled with chitosan for oral insulin delivery.* Biomacromolecules, 2007. **8**(1): p. 146-152.
212. Han L, Zhao Y, Yin L, Li R, Liang Y, Huang H, Pan S, Wu C, Feng M, *Insulin-loaded pH-sensitive hyaluronic acid nanoparticles enhance transcellular delivery.* AAPS PharmSciTech, 2012. **13**(3): p. 836-845.
213. Zhu S, Chen S, Gao Y, Guo F, Li F, Xie B, Zhou J, Zhong H, *Enhanced oral bioavailability of insulin using PLGA nanoparticles co-modified with cell-penetrating peptides and Engrailed secretion peptide (Sec).* Drug Deliv, 2015: p. 1-12.
214. Damgé C, Socha M, Ubrich N, Maincent P, *Poly(ε-caprolactone)/eudragit nanoparticles for oral delivery of aspart-insulin in the treatment of diabetes.* J Pharm Sci, 2010. **99**(2): p. 879-889.
215. Foss AC, Goto T, Morishita M, Peppas NA, *Development of acrylic-based copolymers for oral insulin delivery.* Eur J Pharm Biopharm, 2004. **57**(2): p. 163-169.
216. Thompson CJ, Tetley L, Uchegbu IF, Cheng WP, *The complexation between novel comb shaped amphiphilic polyallylamine and insulin-Towards oral insulin delivery.* Int J Pharm, 2009. **376**(1-2): p. 46-55.
217. Kumari A, Yadav SK, Yadav SC, *Biodegradable polymeric nanoparticles based drug delivery systems.* Colloids Surf B, 2010. **75**(1): p. 1-18.
218. *NanoMega*. Available from: http://www.nanomegamedical.com/.
219. *Aphios Pharma* [cited 2015 Nov 24]; Available from: http://www.aphios.com/.
220. *NOD Pharmaceuticals*. [cited 2015 Nov 24]; Available from: http://www.nodpharm.com/.
221. Lu JM, Wang XW, Marin-Muller C, Wang H, Lin PH, Yao QZ, Chen CY, *Current advances in research and clinical applications of PLGA-based nanotechnology.* Expert Rev Mol Diagn, 2009. **9**(4): p. 325-341.
222. Wu ZM, Zhou L, Guo XD, Jiang W, Ling L, Qian Y, Luo KQ, Zhang LJ, *HP55-coated capsule containing PLGA/RS nanoparticles for oral delivery of insulin.* Int J Pharm, 2012. **425**(1-2): p. 1-8.
223. Reix N, Parat A, Seyfritz E, Van Der Werf R, Epure V, Ebel N, Danicher L, Marchioni E, Jeandidier N, Pinget M, Frère Y, Sigrist S, *In vitro uptake evaluation in Caco-2 cells and in vivo results in diabetic rats of insulin-loaded PLGA nanoparticles.* Int J Pharm, 2012. **437**(1-2): p. 213-220.
224. Santander-Ortega MJ, Bastos-Gonzalez D, Ortega-Vinuesa JL, Alonso MJ, *Insulin-loaded PLGA nanoparticles for oral administration: an in vitro physico-chemical characterization.* J Biomed Nanotechnol, 2009. **5**(1): p. 45-53.
225. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Preat V, *PLGA-based nanoparticles: an overview of biomedical applications.* J Control Release, 2012. **161**(2): p. 505-522.
226. Cui F, Shi K, Zhang L, Tao A, Kawashima Y, *Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: Preparation, in vitro characterization and in vivo evaluation.* J Control Release, 2006. **114**(2): p. 242-50.

227. Jiang T, Singh B, Li H-S, Kim Y-K, Kang S-K, Nah J-W, Choi Y-J, Cho C-S, *Targeted oral delivery of BmpB vaccine using porous PLGA microparticles coated with M cell homing peptide-coupled chitosan.* Biomaterials, 2014. **35**(7): p. 2365-2373.
228. Muller RH, Shegokar R, Keck CM, *20 years of lipid nanoparticles (SLN and NLC): Present state of development and industrial applications.* Curr Drug Discov Technol, 2011. **8**(3): p. 207-227.
229. Mehnert W, Mader K, *Solid lipid nanoparticles - Production, characterization and applications.* Adv Drug Delivery Rev, 2001. **47**(2-3): p. 165-196.
230. Almeida AJ, Souto E, *Solid lipid nanoparticles as a drug delivery system for peptides and proteins.* Adv Drug Delivery Rev, 2007. **59**(6): p. 478-490.
231. Lee JH, Il Ahn S, Park JH, Kim YT, Khang G, Rhee JM, Lee HB, *Solid lipid nanoparticles as a drug delivery system for peptides and proteins.* Tissue Eng Regen Med, 2008. **5**(2): p. 215-228.
232. Sarmento B, Martins S, Ferreira D, Souto EB, *Oral insulin delivery by means of solid lipid nanoparticles.* Int J Nanomedicine, 2007. **2**(4): p. 743-749.
233. Zhang N, Ping Q, Huang G, Xu W, Cheng Y, Han X, *Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin.* Int J Pharm, 2006. **327**(1–2): p. 153-159.
234. Zhang ZH, Zhang YL, Zhou JP, Lv HX, *Solid lipid nanoparticles modified with stearic acid-octaarginine for oral administration of insulin.* Int J Nanomedicine, 2012. **7**: p. 3333-3339.
235. Gao W, Chan JM, Farokhzad OC, *pH-Responsive Nanoparticles for Drug Delivery.* Mol Pharmaceutics, 2010. **7**(6): p. 1913-1920.
236. Zhang H, Liu D, Shahbazi MA, Makila E, Herranz-Blanco B, Salonen J, Hirvonen J, Santos HA, *Fabrication of a multifunctional nano-in-micro drug delivery platform by microfluidic templated encapsulation of porous silicon in polymer matrix.* Adv Mater, 2014. **26**(26): p. 4497-4503.
237. Sheng J, Han L, Qin J, Ru G, Li R, Wu L, Cui D, Yang P, He Y, Wang J, *N-Trimethyl Chitosan Chloride-Coated PLGA Nanoparticles Overcoming Multiple Barriers to Oral Insulin Absorption.* Acs Appl Mater Inter, 2015. **7**(28): p. 15430-15441.
238. Jin Y, Song Y, Zhu X, Zhou D, Chen C, Zhang Z, Huang Y, *Goblet cell-targeting nanoparticles for oral insulin delivery and the influence of mucus on insulin transport.* Biomaterials, 2012. **33**(5): p. 1573-1582.
239. Millotti G, Perera G, Vigl C, Pickl K, Sinner FM, Bernkop-Schnurch A, *The use of chitosan-6-mercaptonicotinic acid nanoparticles for oral peptide drug delivery.* Drug Deliv, 2011. **18**(3): p. 190-197.
240. Sonaje K, Chen YJ, Chen HL, Wey SP, Juang JH, Nguyen HN, Hsu CW, Lin KJ, Sung HW, *Enteric-coated capsules filled with freeze-dried chitosan/poly(gamma-glutamic acid) nanoparticles for oral insulin delivery.* Biomaterials, 2010. **31**(12): p. 3384-3394.
241. Kurita K, *Chitin and chitosan: Functional biopolymers from marine crustaceans.* Mar Biotechnol (NY), 2006. **8**(3): p. 203-226.
242. Benediktsdottir BE, Baldursson O, Masson M, *Challenges in evaluation of chitosan and trimethylated chitosan (TMC) as mucosal permeation enhancers: From synthesis to in vitro application.* J Control Release, 2014. **173**: p. 18-31.
243. Nagpal K, Singh SK, Mishra DN, *Chitosan nanoparticles: A promising system in novel drug delivery.* Chem Pharm Bull, 2010. **58**(11): p. 1423-1430.
244. Kean T, Thanou M, *Biodegradation, biodistribution and toxicity of chitosan.* Adv Drug Delivery Rev, 2010. **62**(1): p. 3-11.
245. Ilium L, *Chitosan and its use as a pharmaceutical excipient.* Pharm Res, 1998. **15**(9): p. 1326-1331.
246. Wedmore I, McManus JG, Pusateri AE, Holcomb JB, *A special report on the chitosan-based hemostatic dressing: Experience in current combat operations.* J Trauma: Inj Infect Crit Care, 2006. **60**(3): p. 655-658.
247. Arai K, Toyosuke Kinumaki, and Takao Fujita, *Toxicity of Chitosan.* Bull Tokai Region Fish Res Lab, 1968. **56**: p. 89-94.
248. Sonaje K, Lin Y-H, Juang J-H, Wey S-P, Chen C-T, Sung H-W, *In vivo evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery.* Biomaterials, 2009. **30**(12): p. 2329-2339.
249. Sogias IA, Williams AC, Khutoryanskiy VV, *Why is chitosan mucoadhesive?* Biomacromolecules, 2008. **9**(7): p. 1837-1842.
250. Illum L, Farraj NF, Davis SS, *Chitosan as a novel nasal delivery system for peptide drugs.* Pharm Res, 1994. **11**(8): p. 1186-1189.

251. Artursson P, Lindmark T, Davis SS, Illum L, *Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2).* Pharm Res, 1994. **11**(9): p. 1358-1361.
252. Yeh T-H, Hsu L-W, Tseng MT, Lee P-L, Sonjae K, Ho Y-C, Sung H-W, *Mechanism and consequence of chitosan-mediated reversible epithelial tight junction opening.* Biomaterials, 2011. **32**(26): p. 6164-6173.
253. Hsu LW, Lee PL, Chen CT, Mi FL, Juang JH, Hwang SM, Ho YC, Sung HW, *Elucidating the signaling mechanism of an epithelial tight-junction opening induced by chitosan.* Biomaterials, 2012. **33**(26): p. 6254-6263.
254. Hsu L-W, Ho Y-C, Chuang E-Y, Chen C-T, Juang J-H, Su F-Y, Hwang S-M, Sung H-W, *Effects of pH on molecular mechanisms of chitosan–integrin interactions and resulting tight-junction disruptions.* Biomaterials, 2013. **34**(3): p. 784-793.
255. Rosenthal R, Gunzel D, Finger C, Krug SM, Richter JF, Schulzke JD, Fromm M, Amasheh S, *The effect of chitosan on transcellular and paracellular mechanisms in the intestinal epithelial barrier.* Biomaterials, 2012. **33**(9): p. 2791-2800.
256. Sarmento B, Ribeiro A, Veiga F, Sampaio P, Neufeld R, Ferreira D, *Alginate/chitosan nanoparticles are effective for oral insulin delivery.* Pharm Res, 2007. **24**(12): p. 2198-2206.
257. Worthington KLS, Adamcakova-Dodd A, Wongrakpanich A, Mudunkotuwa IA, Mapuskar KA, Joshi VB, Guymon CA, Spitz DR, Grassian VH, Thorne PS, Salem AK, *Chitosan coating of copper nanoparticles reduces in vitro toxicity and increases inflammation in the lung.* Nanotechnology, 2013. **24**(39): p. 395101.
258. Prego C, Garcia M, Torres D, Alonso MJ, *Transmucosal macromolecular drug delivery.* J Control Release, 2005. **101**(1-3): p. 151-162.
259. Amidi M, Mastrobattista E, Jiskoot W, Hennink WE, *Chitosan-based delivery systems for protein therapeutics and antigens.* Adv Drug Deliv Rev, 2010. **62**(1): p. 59-82.
260. Foger F, Schmitz T, Bernkop-Schnurch A, *In vivo evaluation of an oral delivery system for P-gp substrates based on thiolated chitosan.* Biomaterials, 2006. **27**(23): p. 4250-4255.
261. Barrett WC, DeGnore JP, Konig S, Fales HM, Keng YF, Zhang ZY, Yim MB, Chock PB, *Regulation of PTP1B via glutathionylation of the active site cysteine 215.* Biochemistry, 1999. **38**(20): p. 6699-6705.
262. Clausen AE, Kast CE, Bernkop-Schnurch A, *The role of glutathione in the permeation enhancing effect of thiolated polymers.* Pharm Res, 2002. **19**(5): p. 602-608.
263. Sakloetsakun D, Iqbal J, Millotti G, Vetter A, Bernkop-Schnurch A, *Thiolated chitosans: Influence of various sulfhydryl ligands on permeation-enhancing and P-gp inhibitory properties.* Drug Dev Ind Pharm, 2011. **37**(6): p. 648-655.
264. Werle M, Hoffer M, *Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine.* J Control Release, 2006. **111**(1–2): p. 41-46.
265. Föger F, Hoyer H, Kafedjiiski K, Thaurer M, Bernkop-Schnürch A, *In vivo comparison of various polymeric and low molecular mass inhibitors of intestinal P-glycoprotein.* Biomaterials, 2006. **27**(34): p. 5855-5860.
266. Dünnhaupt S, Barthelmes J, Köllner S, Sakloetsakun D, Shahnaz G, Düregger A, Bernkop-Schnürch A, *Thiolated nanocarriers for oral delivery of hydrophilic macromolecular drugs.* Carbohyd Polym, 2015. **117**: p. 577-584.
267. Bernkop-Schnurch A, Guggi D, Pinter Y, *Thiolated chitosans: development and in vitro evaluation of a mucoadhesive, permeation enhancing oral drug delivery system.* J Control Release, 2004. **94**(1): p. 177-186.
268. Gradauer K, Barthelmes J, Vonach C, Almer G, Mangge H, Teubl B, Roblegg E, Dünnhaupt S, Fröhlich E, Bernkop-Schnürch A, Prassl R, *Liposomes coated with thiolated chitosan enhance oral peptide delivery to rats.* J Control Release, 2013. **172**(3): p. 872-878.
269. Shi NQ, Qi XR, Xiang B, Zhang Y, *A survey on "Trojan Horse" peptides: Opportunities, issues and controlled entry to "Troy".* J Control Release, 2014. **194**: p. 53-70.
270. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF, *In vivo protein transduction: Delivery of a biologically active protein into the mouse.* Science, 1999. **285**(5433): p. 1569-1572.
271. Khafagy el S, Morishita M, *Oral biodrug delivery using cell-penetrating peptide.* Adv Drug Deliv Rev, 2012. **64**(6): p. 531-539.
272. Liang JF, Yang VC, *Insulin-cell penetrating peptide hybrids with improved intestinal absorption efficiency.* Biochem Biophys Res Commun, 2005. **335**(3): p. 734-738.

273. Kamei N, Morishita M, Ehara J, Takayama K, *Permeation characteristics of oligoarginine through intestinal epithelium and its usefulness for intestinal peptide drug delivery.* J Control Release, 2008. **131**(2): p. 94-99.
274. Steinbach JM, Seo Y-E, Mark Saltzman W, *Cell Penetrating Peptide-Modified Poly(Lactic-co-Glycolic Acid) Nanoparticles with Enhanced Cell Internalization.* Acta Biomaterialia. **30**: p. 49-61.
275. Guo F, Zhang M, Gao Y, Zhu S, Chen S, Liu W, Zhong H, Liu J, *Modified nanoparticles with cell-penetrating peptide and amphipathic chitosan derivative for enhanced oral colon absorption of insulin: preparation and evaluation.* Drug Deliv, 2015: p. 1-12.
276. Rahmat D, Khan MI, Shahnaz G, Sakloetsakun D, Perera G, Bernkop-Schnürch A, *Synergistic effects of conjugating cell penetrating peptides and thiomers on non-viral transfection efficiency.* Biomaterials, 2012. **33**(7): p. 2321-2326.
277. Yoshida T, Lai TC, Kwon GS, Sako K, *pH- and ion-sensitive polymers for drug delivery.* Expert Opin Drug Deliv, 2013. **10**(11): p. 1497-1513.
278. Tanno F, Nishiyama Y, Kokubo H, Obara S, *Evaluation of hypromellose acetate succinate (HPMCAS) as a carrier in solid dispersions.* Drug Dev Ind Pharm, 2004. **30**(1): p. 9-17.
279. Sarode AL, Obara S, Tanno FK, Sandhu H, Iyer R, Shah N, *Stability assessment of hypromellose acetate succinate (HPMCAS) NF for application in hot melt extrusion (HME).* Carbohyd Polym, 2014. **101**: p. 146-153.
280. Vasiliauskas R, Liu D, Cito S, Zhang H, Shahbazi M-A, Sikanen T, Mazutis L, Santos HA, *Simple microfluidic approach to fabricate monodisperse hollow microparticles for multidrug delivery.* Acs Appl Mater Inter, 2015. **7**(27): p. 14822-14832.
281. Wu C-s, Wang X-q, Meng M, Li M-g, Zhang H, Zhang X, Wang J-c, Wu T, Nie W-h, Zhang Q, *Effects of pH-sensitive nanoparticles prepared with different polymers on the distribution, adhesion and transition of Rhodamine 6G in the gut of rats.* J Microencapsul, 2010. **27**(3): p. 205-217.
282. Wang XQ, Zhang Q, *pH-sensitive polymeric nanoparticles to improve oral bioavailability of peptide/protein drugs and poorly water-soluble drugs.* Eur J Pharm Biopharm, 2012. **82**(2): p. 219-229.
283. Verma A, Sharma S, Gupta PK, Singh A, Teja BV, Dwivedi P, Gupta GK, Trivedi R, Mishra PR, *Vitamin B12 functionalized layer by layer calcium phosphate nanoparticles: A mucoadhesive and pH responsive carrier for improved oral delivery of insulin.* Acta Biomaterialia. **31**: p. 288-300.
284. Choi SR, Jang DJ, Kim S, An S, Lee J, Oh E, Kim J, *Polymer-coated spherical mesoporous silica for pH-controlled delivery of insulin.* J Mater Chem B, 2014. **2**(6): p. 616-619.
285. Wang Y, Dave RN, Pfeffer R, *Polymer coating/encapsulation of nanoparticles using a supercritical anti-solvent process.* J Supercrit Fluids, 2004. **28**(1): p. 85-99.
286. Liu D, Zhang H, Herranz-Blanco B, Makila E, Lehto VP, Salonen J, Hirvonen J, Santos HA, *Microfluidic assembly of monodisperse multistage pH-responsive polymer/porous silicon composites for precisely controlled multi-drug delivery.* Small, 2014. **10**(10): p. 2029-2038.
287. Rahikkala A, Junnila S, Vartiainen V, Ruokolainen J, Ikkala O, Kauppinen E, Raula J, *Polypeptide-based aerosol nanoparticles: Self-assembly and control of conformation by solvent and thermal annealing.* Biomacromolecules, 2014. **15**(7): p. 2607-2615.
288. Sapra M, Pawar AA, Venkataraman C, *A single-step aerosol process for in-situ surface modification of nanoparticles: Preparation of stable aqueous nanoparticle suspensions.* J Colloid Interface Sci, 2016. **464**: p. 167-174.
289. Salonen J, Mäkilä E, Riikonen J, Heikkilä T, Lehto VP, *Controlled enlargement of pores by annealing of porous silicon.* Phys Status Solidi A, 2009. **206**(6): p. 1313-1317.
290. Hillamo RE, Kauppinen EI, *On the Performance of the Berner Low-Pressure Impactor.* Aerosol Sci. Technol., 1991. **14**(1): p. 33-47.
291. Brunauer S, Emmett PH, Teller E, *Adsorption of gases in multimolecular layers.* J Am Chem Soc, 1938. **60**(2): p. 309-319.
292. Antunes F, Andrade F, Araujo F, Ferreira D, Sarmento B, *Establishment of a triple co-culture in vitro cell models to study intestinal absorption of peptide drugs.* Eur J Pharm Biopharm, 2013. **83**(3): p. 427-435.
293. Araújo F, Sarmento B, *Towards the characterization of an in vitro triple co-culture intestine cell model for permeability studies.* Int J Pharm, 2013. **458**(1): p. 128-134.

294. Nakamura T, Terajima T, Ogata T, Ueno K, Hashimoto N, Ono K, Yano S, *Establishment and pathophysiological characterization of type 2 diabetic mouse model produced by streptozotocin and nicotinamide.* Biol Pharm Bull, 2006. **29**(6): p. 1167-1174.
295. Szkudelski T, *Streptozotocin-nicotinamide-induced diabetes in the rat. Characteristics of the experimental model.* Exp Biol Med (Maywood), 2012. **237**(5): p. 481-490.
296. Ghasemi A, Khalifi S, Jedi S, *Streptozotocin-nicotinamide-induced rat model of type 2 diabetes (review).* Acta Vet Hung, 2014. **101**(4): p. 408-420.
297. Nel AE, Madler L, Velegol D, Xia T, Hoek EMV, Somasundaran P, Klaessig F, Castranova V, Thompson M, *Understanding biophysicochemical interactions at the nano-bio interface.* Nat Mater, 2009. **8**(7): p. 543-557.
298. Cohen-Sela E, Chorny M, Koroukhov N, Danenberg HD, Golomb G, *A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles.* J Control Release, 2009. **133**(2): p. 90-95.
299. Chakravarthi SS, Robinson DH, *Enhanced cellular association of paclitaxel delivered in chitosan-PLGA particles.* Int J Pharm, 2011. **409**(1-2): p. 111-120.
300. Gupta S, Jain A, Chakraborty M, Sahni JK, Ali J, Dang S, *Oral delivery of therapeutic proteins and peptides: a review on recent developments.* Drug Deliv, 2013. **20**(6): p. 237-246.
301. Li J, Jiang GQ, Ding FX, *The effect of pH on the polymer degradation and drug release from PLGA-mPEG microparticles.* J Appl Polym Sci, 2008. **109**(1): p. 475-482.
302. Sarmento B, Andrade F, da Silva SB, Rodrigues F, das Neves J, Ferreira D, *Cell-based in vitro models for predicting drug permeability.* Expert Opin Drug Metab Toxicol, 2012. **8**(5): p. 607-621.
303. Huhn D, Kantner K, Geidel C, Brandholt S, De Cock I, Soenen SJ, Rivera Gil P, Montenegro JM, Braeckmans K, Mullen K, Nienhaus GU, Klapper M, Parak WJ, *Polymer-coated nanoparticles interacting with proteins and cells: focusing on the sign of the net charge.* ACS Nano, 2013. **7**(4): p. 3253-3263.
304. Fonte P, Andrade JC, Seabra V, Sarmento B, *Chitosan-based nanoparticles as delivery systems of therapeutic proteins.* Methods Mol Biol, 2012. **899**: p. 471-487.
305. Kojima H, Yoshihara K, Sawada T, Kondo H, Sako K, *Extended release of a large amount of highly water-soluble diltiazem hydrochloride by utilizing counter polymer in polyethylene oxides (PEO)/polyethylene glycol (PEG) matrix tablets.* Eur J Pharm Biopharm, 2008. **70**(2): p. 556-562.
306. Hans ML, Lowman AM, *Biodegradable nanoparticles for drug delivery and targeting.* Curr Opin Solid St M, 2002. **6**(4): p. 319-327.
307. Fernandes M, Goncalves IC, Nardecchia S, Amaral IF, Barbosa MA, Martins MCL, *Modulation of stability and mucoadhesive properties of chitosan microspheres for therapeutic gastric application.* Int J Pharm, 2013. **454**(1): p. 116-124.
308. Sonaje K, Lin K-J, Tseng MT, Wey S-P, Su F-Y, Chuang E-Y, Hsu C-W, Chen C-T, Sung H-W, *Effects of chitosan-nanoparticle-mediated tight junction opening on the oral absorption of endotoxins.* Biomaterials, 2011. **32**(33): p. 8712-8721.
309. Araujo F, Shrestha N, Granja PL, Hirvonen J, Santos HA, Sarmento B, *Antihyperglycemic potential of incretins orally delivered via nano and microsystems and subsequent glucoregulatory effects.* Curr Pharm Biotechnol, 2014. **15**(7): p. 609-619.
310. Trapani A, Palazzo C, Contino M, Perrone MG, Cioffi N, Ditaranto N, Colabufo NA, Conese M, Trapani G, Puglisi G, *Mucoadhesive properties and interaction with P-glycoprotein (P-gp) of thiolated-chitosans and -glycol chitosans and corresponding parent polymers: a comparative study.* Biomacromolecules, 2014. **15**(3): p. 882-893.
311. Sakloetsakun D, Bernkop-Schnürch A, *Thiolated chitosans.* J Drug Deliv Sci Technol, 2010. **20**(1): p. 63-69.
312. Mislick KA, Baldeschwieler JD, *Evidence for the role of proteoglycans in cation-mediated gene transfer.* Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12349-12354.
313. Wang LH, Rothberg KG, Anderson RG, *Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation.* J Cell Biol, 1993. **123**(5): p. 1107-1117.
314. Sai Y, Kajita M, Tamai I, Wakama J, Wakamiya T, Tsuji A, *Adsorptive-mediated endocytosis of a basic peptide in enterocyte-like Caco-2 cells.* Am J Physiol, 1998. **275**(3 Pt 1): p. G514-520.
315. Schnitzer JE, Oh P, Pinney E, Allard J, *Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules.* J Cell Biol, 1994. **127**(5): p. 1217-1232.

Recent Publications in this Series

2/2016 Sanna Toivonen
Derivation of Hepatocyte Like Cells from Human Pluripotent Stem Cells
3/2016 Marjaana Peltola
AMIGO-Kv2.1 Potassium Channel Complex: Identification and Association with Schizophrenia-Related Phenotypes
4/2016 Niko-Petteri Nykänen
Cellular Physiology and Cell-to-Cell Propagation of Tau in Neurodegeneration: The Impact of Late-Onset Alzheimer's Disease Susceptibility Genes
5/2016 Liisa Korkalo
Hidden Hunger in Adolescent Mozambican Girls: Dietary Assessment, Micronutrient Status, and Associations between Dietary Diversity and Selected Biomarkers
6/2016 Teija Ojala
Lactobacillus crispatus and *Propionibacterium freudenreichii*: A Genomic and Transcriptomic View
7/2016 César Araujo
Prostatic Acid Phosphatase as a Regulator of Endo/Exocytosis and Lysosomal Degradation
8/2016 Jens Verbeeren
Regulation of the Minor Spliceosome through Alternative Splicing and Nuclear Retention of the U11/U12-65K mRNA
9/2016 Xiang Zhao
HMGB1 (Amphoterin) and AMIGO1 in Brain Development
10/2016 Tarja Pääkkönen (Jokinen)
Benign Familial Juvenile Epilepsy in Lagotto Romagnolo Dogs
11/2016 Nora Hiivala
Patient Safety Incidents, Their Contributing and Mitigating Factors in Dentistry
12/2016 Juho Heinonen
Intravenous Lipid Emulsion for Treatment of Local Anaesthetic and Tricyclic Antidepressant Toxicity
13/2016 Riikka Jokinen
Genetic Studies of Tissue-Specific Mitochondrial DNA Segregation in Mammals
14/2016 Sanna Mäkelä
Activation of Innate Immune Responses by Toll-like Receptors and Influenza Viruses
15/2016 Mari Hirvinen
Immunological Boosting and Personalization of Oncolytic Virotherapies for Cancer Treatment
16/2016 Sofia Montalvão
Screening of Marine Natural Products and Their Synthetic Derivatives for Antimicrobial and Antiproliferative Properties
17/2016 Mpindi John Patrick
Bioinformatic Tools for Analysis, Mining and Modelling Large-Scale Gene Expression and Drug Testing Datasets
18/2016 Hilla Sumanen
Work Disability among Young Employees Changes over Time and Socioeconomic Differences
19/2016 Oyediran Olulana Akinrinade
Bioinformatic and Genomic Approaches to Study Cardiovascular Diseases
20/2016 Prasanna Sakha
Development of Microfluidic Applications to Study the Role of Kainate Receptors in Synaptogenesis

Helsinki 2016 ISSN 2342-3161 ISBN 978-951-51-2039-7