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Nanotechnological Strategies for Coating and Encapsulation of Therapeutic Proteins

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Nanotechnological Strategies for Coating and Encapsulation of Therapeutic Proteins

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LIST OF ABREVIATIONS

ACN	Acetonitrile
ATCC	American Type Culture Collection
BBB	Blood-Brain Barrier
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CV	Column Volumes
DA	Dopaminergic
DMEM	Dulbecco's Modified Eagle Medium
EA	Enzimatic Activity
EE	Encapsulation Efficiency
EMA	European Medicine Agency
ER	Endoplasmic Reticulum
ERT	Enzyme Replacement Therapy
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
Gcase	Glucosylceramidase beta
GD	Gaucher Disease
hGBA	Human Glucocerebrosidase (or β-Glucocerebrosidase)
HGMD	Human Gene Mutation database
HGNC	HUGO Gene Nomenclature Committee
IV	Intravenous
LB	Lewy Body
LSD	Lysosomal Storage Disease
LZM	Lysozyme
MEM	Minimum Essential Medium
MEMα-Ø	MEMα supplemented with GlutaMAX without FBS
MRT	Mean Residence Time
MWCO	Molecular Weight Cut-Off
NHS	N-hydroxysuccinimide
NvCI	*Nerita versicolor* Carboxipeptidase Inhibitor
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PD	Parkinson's Disease
PEG	Polyethylene Glycol
PES	Polyethersulfone
PGA	Poly(L-glutamic acid)
PGA-PD	Pyridyl Disulfide Modified PGA
PLGA	Poly(D,L-lactide-co-glycolide)
PVDF	Polyvinylidene Fluoride
rhEGF	recombinant human Epithelial Growth Factor

RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
SATP	N-Succinimidyl S-Acetylthiopropionate
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SRT	Substrate Reduction Therapy
TFA	Trifluoroacetic Acid
w/o/w	Water in oil in water

Chapter I:

Introduction

CHAPTER I: INTRODUCTION

1 Nanotechnology

1.1 "There's Plenty of Room at the Bottom"

On the 29th of December, 1959, the Physicist Richard Feynman gave the famous lecture "There's Plenty of Room at the Bottom". In front of the American Physical Society, the 1965 Nobel Prize Laureate introduced the concept of manipulating matter at the atomic level, predicting that the development of more precise microscopes would allow us to access the field of individual atoms and theorising about the production and control of tiny machines on the basis of quantum mechanics. Although in his talk Feynman didn't use the term "nano" whatsoever, and the fact that the talk itself remained unnoticed for a couple of decades, Feynman is anyway considered the father of Modern Nanotechnology, since his hypothesis have been proven correct so far (Hulla, Sahu and Hayes, 2015; Krukemeyer et al., 2015)

Although there is no consensus for a definition of Nanotechnology as it is an evolving field, the European Commission has suggested the following definition:

> "Nanotechnology is the understanding and control of matter at dimensions between approximately 1 and 100 nanometres, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modelling, and manipulating matter at this length scale."

Nonetheless, nanotechnology as human manipulation of matter at the nanoscale is not a new field, even if unknowingly. Earlier evidences in the use of nanomaterials start around 600 BC, and some examples are the presence of nanometre-size particles of carbon in Damascene Swords, or the optical effects seen in the Lycurgus Cup, an antique Roman from the 4th Century AD. We now know that the presence of carbon nanoparticles in steel alloys is responsible for the elasticity and resistance of Damascene Steel; and that the presence of colloidal nanoparticles of silver and gold contained in glass are responsible for the optical effects observed in the Lycurgus Cup (Krukemeyer et al., 2015).

The development of humankind has always been tracked by its knowledge on the manipulation of certain materials, with early humans advancing from stone to bronze and iron over the expanse of several thousand years. The revolution in materials is still ongoing, with

one of the primary objectives being to engineer at the nanoscale in order to exploit novel material properties of that magnitude. Experts in the field of nanoscience can agree that the onset of the nano-age is not yet upon us, but short-term and long-term advances may one day bring applied nanomaterials to the everyday human-material interchange. Even now, more than 1500 consumer products have been manufacture-identified as involving nanotechnology (Vance et al., 2015).

The term Nanotechnology itself was first used by the Japanese scientist Norio Taniguchi and popularised by Eric Drexler in the 1986 book "Engines of Creation: The Coming Era of Nanotechnology". It is around the 1980s when it is considered that the golden era of nanotechnology started. At this point nanotechnology is considered one of the key technologies of the 21st century, and is at the meeting point of physics, chemistry, biology, medicine, electronics and information technology. Concerning natural sciences, the boundaries with nanotechnology are blurry and overlap in many areas, such as pharmaceuticals, biotechnology, diagnostics, tissue engineering, medical devices and medical devices design (Krukemeyer et al., 2015; Bheemidi et al., 2011).

Nanobiotechnology focuses in the study of molecular intra- and intercellular processes, and tries to establish its importance for nanotechnology applications in medicine. The interrelation between nanobiotechnology and nanomedicine groups many fields in the area of life sciences, material sciences, chemistry and medicine, such as nanobiotechnology, gene and cell therapy, drug delivery and biomarkers amongst others; as shown in Figure 1.1. Those fields converge in the development of a new discipline that creates novel and innovative methods and inventions for therapy and medical diagnosis (Jain, 2008).

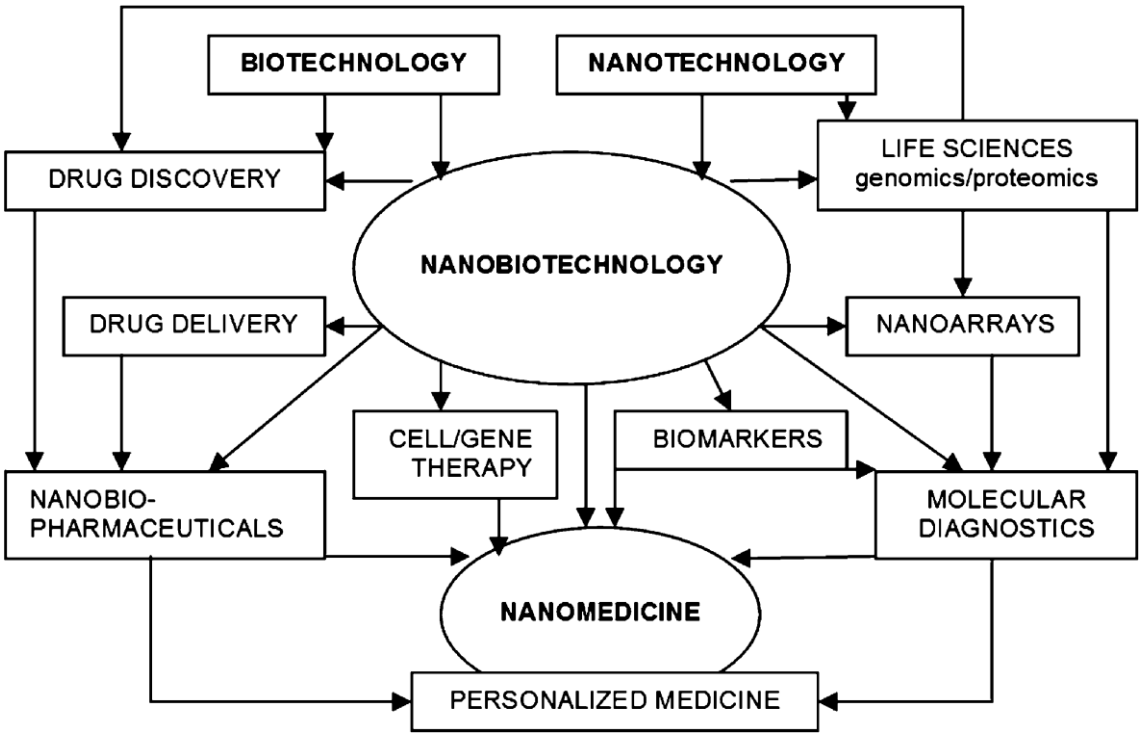

Figure 1.1. Relationship of nanobiotechnology and nanomedicine. Schematic representation obtained from the Handbook of Nanomedicine (Jain, 2008).

1.2 Nanomedicine

One of the areas where nanomaterials is expected to transform is medicine, thus creating the field of nanomedicine. The predicted potential of nanomaterials in medicine is wide-range, covering both disease diagnosis and treatment. Drug delivery is deemed as a promising domain for use of nanomaterials such as nanoparticles or even nanotubes to act as vehicles for carrying drugs to targeted sites in the body (Krukemeyer et al., 2015). It is also important to note that nanomedicine does not only consider inorganic or metallic nanomaterials, but a bulk of studies focus on polymeric, liposomal or protein based nanoparticles, also classed as such because of their size. Nanoparticles also show promise as MRI contrast agents that can enhance medical imaging. Furthermore, thanks to their mechanical properties, nanomaterials are considered for growing cell and tissue scaffolds. There is also a capacity for them to be used in wear-resistant, biocompatible medical implants. Further development in nanoelectronics opens up the possibility of bio-sensors or even artificial retinas or cochleae. These applications are more complex than other already established uses, such as titanium dioxide or zinc oxide nanoparticle incorporation into sunscreens to protect skin from UV light irradiation.

It is evident that the nanomedicine age has not fully materialised as many listed potential applications have yet to become market products. The field is still nascent but growing exponentially, with FDA registered trials of novel nanomedicines increasing threefold within the recent 3-year period. Such numbers also motivate competition within the industry. In fact, nanomedicine is a young scientific discipline originated around the 1990s with the intent to unveil how nanotechnology can be of use to medicine, medical technology and pharmacology. These possible uses are contemplated in three main pillars: (1) nanomaterials and nanoconstructs that can be used as biosensors, as aids in treatment and as transporters; (2) knowledge of molecular medicine (covering the fields of genetics, proteomics and microorganisms synthetically produced or modified); and (3) nanotechnologies for rapid diagnosis, therapy, repair, cell surgery or improving physiological functions (Krukemeyer et al., 2015). The possible interactions between nanotechnology and medicine are vast and only limited by human imagination. Expectations for fields like diagnostics, therapeutics and regenerative medicine are immense.

It is of particular interest in the pharmacology field and for this particular project all the potential applications of nanotechnology on the field of therapeutics and drug delivery, with a particular focus on protein therapeutics. The existence of many limitations in the use of proteins in therapies makes the irruption of nanomedicine a great opportunity for improvements in that field.

1.3 Protein therapeutics

Proteins, as the most abundant biological group of macromolecules, mediate virtually every process that takes place in a cell. They are the molecular instrument by which genetic information is expressed, and display and endless diversity for an endless diversity of functions. Amongst them, enzymes are the reaction catalyst of biological systems, in charge of catalysing almost every biochemical reaction. Enzymes are powerful and specific catalysts, and their properties are tightly connected with their respective structure (Nelson, Lehninger and Cox, 2008).

Proteins are polymers made up of a set of 20 amino acids linked covalently in a linear sequence. Amino acids share common structural features but display side chains with distinctive chemical properties. The chemical interactions of polypeptide chains, internal and

with the environment, explain part of the folding process. As proteins structures have evolved to function in particular environments, variations in those conditions can result in protein structural changes, and subsequent alterations of the protein activity (Nelson, Lehninger and Cox, 2008).

In a simplification of how enzymes work, these catalysts enhance reaction rates by forming a complex between them and the substrate, lowering the activation energy of the reaction. Weak interactions are formed between the enzyme and the substrate (mainly hydrogen bonds and ionic and hydrophobic interactions) (Nelson, Lehninger and Cox, 2008).

Environment condition changes such as pH, temperature, ionic strength or the presence of hydrophobic substances may alter the protein by altering protonation, charge density distribution or breaking weak bonds. Those changes may affect the capacity of the enzyme to form a complex with the substrate either by impossibility to bond or because of lack of complementarity, or may disrupt the ability to catalyse the reaction itself. A simple change in the protonation of a side chain in the active site can be enough to prevent the reaction progression. The presence of organic solvents and other hydrophobic substances alter the hydrophobic interaction within the protein and between the protein and the environment, leading to protein unfold (Nelson, Lehninger and Cox, 2008; Grahame, Bryksa and Yada, 2015).

Protein-based therapeutics have had an important impact in the treatment and management of many human diseases in the last three decades. A wide variety of treatments and drugs of protein or peptide nature, such as enzymes, antibodies and hormones amongst others have been approved and become effective therapies for a myriad of human diseases including diabetes, cancer and Lysosomal Storage Diseases (LSD) (Yu et al., 2016; Marcucci et al., 2014). The advent of DNA technology and the incessant discoveries and advances in the field have a key role in the surge of protein therapeutics, immensely reducing time and resources in protein production and availability. Back in 1982, the Food and Drug Administration (FDA) approved the first commercially available recombinant therapeutic protein, Human insulin, which became the major therapy for diabetes mellitus type I and II (Yu et al., 2016).

Inherited enzyme deficiencies are genetic disorders in which a defective gene results in a defective enzyme, with subsequent metabolic consequences. Examples of these disorders are the Lysosome Storage Diseases (LSD), illnesses caused by mutations in lysosomal acid hydrolases, receptors, and activator proteins among others, which result in accumulation of substrates. Many Enzyme Replacement Therapies (ERT) have been developed over the years intended to replace the deficient enzymes from these disorders with recombinant, wild type

ones able to restore their activity. Specifics of ERT in LSD will be discussed later on (Brady, 2006).

Protein therapeutics offer many advantages when compared with conventional, small-molecular drugs, such as higher specificity, greater activity and lower toxicity levels. However, many important shortcomings affect therapeutic proteins that significantly affect their efficiency, stability and in the end, their therapeutic capacity. Proteins are sensitive to enzymatic degradation, have short circulation half-lives in the bloodstream, and its delivery to targeted sites can be severely affected by the poor membrane permeability of proteins. Additional body barriers, like the Blood-Brain Barrier (BBB), forbid the proteins unable to cross them to reach several regions of the bodies. Proteins also present structural complexity, necessary for activity performance and specificity maintenance. The need to maintain its structure pose a difficulty to protein modification if needed, and makes it vulnerable to environmental conditions that challenge protein conformation. Also, improper design or formulation of protein drug, besides affecting its activity and specificity, may cause degradation, denaturation or aggregation (Yu et al., 2016).

In order to overcome the limitations of protein therapies and improve their therapeutic efficacy, nanotechnology offers very promising tools and many approaches have already been made, such as encapsulation with micro- and nanoparticles, chemical modification with hydrophilic polymers and protein engineering.

1.4 Nanotechnology-based drug delivery systems for proteins

Sensitivity of proteins to environmental conditions, including temperature, pH, Ionic strength, preservatives and surfactants, represents a challenge when it comes to leverage their therapeutic properties. The lack of appropriate pharmacokinetics of peptides and proteins can also be limiting for practical uses. In recent years, the use of vehicles to overcome protein limitations as a therapeutic agent have been studied (Mohammadi-Samani and Taghipour, 2015).

Nanoparticles like liposomes, micelles, polymer nanoparticles and inorganic materials has been proven useful as drug carriers, and many advantages have been described in using them protein encapsulation: (1) protection against degradation in biological environment, (2) increase of protein half-life in systemic circulation, (3) ability to maintain a sustained release

and (4) target tissues, cells or intracellular compartments for a safer and more effective delivery (Yu et al., 2016).

1.4.1 PLGA nanoparticles

Poly(lactic-*co*-glycolic acid) (PLGA) is one of the most used polymers for nanoparticle production due to its properties. PLGA is biocompatible, biodegradable, possesses good mechanical strength, show good sustained release properties and its use has already been approved by the FDA and the EMA. Many natural and synthetic polymers have been studied as vehicles for therapeutic proteins, and although many natural are in use for oral delivery, only synthetic polymers, with PLGA amongst them, have the FDA authorisation to be used in parenteral administration (Mohammadi-Samani and Taghipour, 2015; Fonte et al., 2014).

PLGA possesses a great flexibility in processing, being able to be used as microspheres, microcapsules, nanoparticles, implants and films. The widely extended use in micro- and nanoparticles includes many observed advantages: (1) reduction of the administration frequency because of the sustained release properties, (2) reduction in protein dosage as continuous release prevents a rapid clearance or degradation, (3) reduction of drug fluctuation in systemic circulation and its subsequent adverse reactions due to sustained and controlled release properties (Mohammadi-Samani and Taghipour, 2015).

Degradation of PLGA, by hydrolysis of the ester linkage between monomers, leads to the formation of lactic and glycolic acid, both assimilable by normal metabolic pathways, without formation of any toxic by-product. PLGA degradation rate can be tuned according the molar ratio of lactic/glycolic acid in the co-polymer structure. The higher the proportion in favour of lactic acid, the more it takes for the co-polymer to be degraded. Environmental pH is also an important factor that influences PLGA degradation (Mohammadi-Samani and Taghipour, 2015).

1.4.1.1 Manufacturing of PLGA nanoparticles. Double emulsion/solvent evaporation method

Due to PLGA processing flexibility, many methods can be chosen for nanoparticle synthesis. The type of polymer, the drug to encapsulate and the final use of the nanoparticle must be taken into account in order to choses an adequate solution. For hydrophilic drugs such as peptides and proteins, one of the best and most used method is the double emulsion/solvent

evaporation method that is easily implementable in the laboratory and scalable (Mohammadi-Samani and Taghipour, 2015).

The solvent evaporation method is based on the formation of an emulsion between a hydrophilic phase (W_1) and a volatile organic solvent (O), which is later evaporated. The protein to encapsulate is dissolved in an aqueous buffer and emulsified in a polymeric solution. The polymeric solutions contains the polymer, PLGA dissolved in an organic solvent solution being dichloromethane and ethyl acetate the most common. The use of appropriate surfactants in the oil phase, such as Span 80, is optional but helps stabilizing the emulsion. The second emulsion is formed by homogenization of the primary emulsion (W_1/O) in a second hydrophilic phase (W_2) containing stabilizer such as polyvinyl alcohol (PVA). The second emulsion (W_1/O/W_2) undergoes magnetic stirring or rotary evaporation so that the organic solvent is evaporated. By evaporation of the organic solvent solid nanoparticles are formed. Surfactant and solvent residuals are removed by centrifugation (Taghipour et al., 2014; Mohammadi-Samani and Taghipour, 2015; Fonte et al., 2014).

The properties of PLGA nanoparticles are affected by many factors tuneable during the synthesis procedure (figure 1.2). A meticulous choice must be made of the different parameters of the synthesis such as (1) the type of organic solvent, (2) the type of polymer (its composition and molecular weight must be considered), (3) the concentration of the polymer, (4) the type and concentration of the surfactant, (5) the method, intensity and duration of homogenization, (6) the ratio among the phases, (7) the properties of the inner face (concentration of the payload and buffer properties), (8) the use of stabilizers in the different phases and a long *etcetera*. The choice of this parameters directly affect the final outcome, modulating (i) the particle size and size distribution, (ii) the surface charge, (iii) the polydispersion, (iv) the encapsulation efficiency and (v) the release profile amongst others (Mohammadi-Samani and Taghipour, 2015).

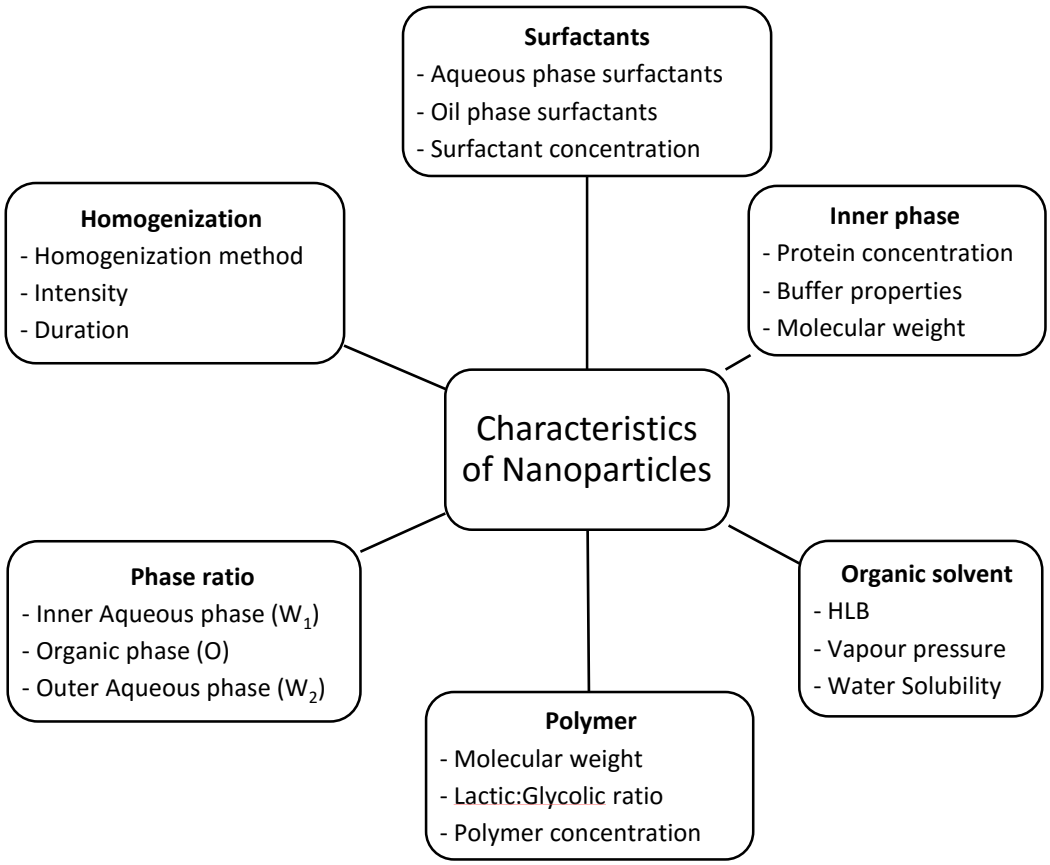

Figure 1.2. Schematics of factors affecting PLGA nanoparticles characteristics. Scheme adapted from Mohammadi-Samani and Toghipour, 2015.

1.4.1.2 Limitations of protein encapsulation in PLGA

Despite all the advantages described for protein encapsulation in PLGA nanoparticles, there are some major shortcomings that can seriously affect the characteristics of the resultant nanoparticles, especially regarding the encapsulated protein biological activity.

During the synthesis process of the nanoparticles, many of the steps that take place can destabilize the protein. The major threat for protein stability is in fact the interface between water and the organic solvent. As introduced before, when in presence of organic solvents, the hydrophobic side chains of a protein, usually protected inside the protein structure, can be exposed to the environment, and the protein denatures and tends to aggregate and experience non-specific adsorption. Homogenization in the first emulsification is also reported to participate in unfolding and aggregation of proteins (Mohammadi-Samani and Taghipour, 2015). The effect on protein stability because of the encapsulation process varies greatly regarding on the protein properties. Proteins with intrinsic enzymatic activity, dependant of

protein conformation, may completely loose its function because of denaturation and unfolding, while a protein ligand with no enzymatic activity may afford unfolding. The size and the presence of disulphide bonds in a protein are two major factors that determine its folding and stability (De Sancho, Doshi and Munoz, 2009; Zavodszky et al., 2001)

Due to PLGA natural occurring hydrolysis into lactic and glycolic acids, PLGA nanoparticles' inner phase display an acidic pH. If not properly buffered, or if the buffer capacity is exceeded, acidification of the inner phase can lead to protein inactivation or denaturation.

Another undesirable effect in PLGA nanoparticles is the burst release, in which a large amount of encapsulated protein, ineffective or damaged in most of the cases, is released within a short time. Burst release can occur via surface absorbed protein and mostly because of the presence of cracks and pores in the nanoparticle (Yu et al., 2016; Mohammadi-Samani and Taghipour, 2015).

1.4.2 Chemical modification of proteins

The short half-life of proteins in systemic circulation is one of the major limitations of protein therapeutics. Not only proteins are vulnerable to enzymatic degradation or inactivation because of the environmental conditions, but also they tend to be cleared from circulation. Proteins under 70 kDa are mostly removed from circulation by glomerular filtration (Yu et al., 2016). Many methods consisting in modification of the therapeutic proteins have been suggested. The attachment of hydrophilic polymers increase their molecular weight and its hydrodynamic radius, being polyethylene glycol (PEG) a prominent example that has been able to reach the market. Other modifications as hyperyglycosylation or conjugation with biodegradable polymers such as Poly-L-glutamic acid (PGA) present advantages over PEG because of its biodegradability, as they can naturally be assimilated by natural metabolic reactions. Additionally, protein coating or conjugation can have a protective effect in the protein from the conditions of the environment (Yu et al., 2016; Talelli and Vicent, 2014).

Protein conjugation with polymers can also be enhanced by the use of specific linkers. The use of linkers sensitive to pH, reductive environment or other environmental conditions allow to keep a protein conjugated in the conditions it is needed, and release it when it reaches its target. A protein targeted towards the lysosome but unstable in systemic circulation, could be conjugated with a polymer via a reduction sensitive linker that would be stable in the oxidizing conditions of the bloodstream, thus protecting the coated protein; but once it reaches the

highly reductive conditions of the lysosome the linker breaks and the protein is released (Talelli and Vicent, 2014).

Chemical modification of proteins can improve their stability and pharmacokinetic properties, but it can also produce significant changes on their conformation and thus cause activity loss, or even toxicity.

2 Peptic Ulcer disease

2.1 Epidemiology and current treatment

The concept peptic ulcer is used for peptic acid injuries in the digestive tract, mainly in the stomach or the proximal duodenum. Those injuries result in mucosal breaks that reach the submucosa. The main risk factors of both gastric and duodenal ulcers are *Helicobacter pylori* and the use of nonsteroidal anti-inflammatory drugs (NSAIDs) or aspirins, although individual susceptibility to bacterial virulence and drug toxicity is suggested to be essential to mucosal damage initiation as only few people under these two conditions end up developing peptic ulcers (Lanas and Chan, 2017).

Recent estimations of peptic ulcer disease prevalence in general population establish an incidence between 0.1 and 0.3% per year, with a lifetime prevalence between 5 and 10%. However, epidemiological studies indicate an abrupt decreasing trend in incidence, hospital admissions and death related to peptic ulcers; a decrease that could be attributable to the introduction of new therapies, the reduction of *H. pylori* infection prevalence or to a generational trend change in the disease prevalence, which is usual in many gastrointestinal diseases. The widespread use of antisecretory drugs and a rationalization in the use of NSAIDs might also have a role in ulcer-related mortality reduction. With the declining of *H. pylori* infection prevalence some studies suggest an increase in idiopathic ulcers (non-NSAID, non-*H. pylori* ulcers). It has been reported that psychological stress and life-threatening conditions could lead to non-NSAID, non-*H. pylori* ulcers (Lanas and Chan, 2017; Yuan, Padol and Hunt, 2006).

Although having small effect in the healing of the ulcers alone, eradication of *H. pylori* is the only effective treatment for *H. pylori* related gastric and duodenal ulcers. Healing of peptic ulcers, either in *H. pylori* related ulcers and in NSAID-related ulcers is managed with

administration of Proton-Pump Inhibitors (PPI), a drug that inhibits gastric acid secretion and ensures high healing rates for peptic ulcers (Yuan, Padol and Hunt, 2006). For idiopathic peptic ulcers, however, higher risk of complicated ulcer disease and poor response to PPI are expected, so a case-by-case management is required in this situation (Chung, Chiang and Lee, 2015).

According to the state of the art, there are some challenges need to be addressed regarding peptic ulcers treatment. Among them, the growing prevalence of antibiotic resistance pose a major threat to eradication of *H. pylori*, and thus the healing of bacterial infection-related ulcers; prevention of ulcer recurrence is considered key in the reduction of morbidity and mortality (Lanas and Chan, 2017). Concerning idiopathic peptic ulcers, establishing the best way to manage non-NSAID, non-*H. pylori*-associated ulcers requires further investigation (Yuan, Padol and Hunt, 2006).

2.2 Role of Epidermal Growth Factor in Peptic Ulcer Healing. A discontinued research line

Several studies carried out around the 1990s decade have established an important role of growth factors, such as Epidermal Growth Factor (EGF) in Peptic Ulcer Healing. Ulcer healing is a complex process which involves many molecular mechanisms such as proliferation of migration of a variety of cells (epithelial and endothelial cells, fibroblasts and macrophages), deposition and organization of extracellular matrix, and differentiation of regenerated tissues (Calabrò et al., 1995). EGF in the gastrointestinal lumen is described to be secreted by the salivary glands and the Brunner's glands of the duodenum , but there is also EGF expression at the margin of the ulcers (Calnan et al., 2000; Brzozowski et al., 2001)

The effects of EGF increasing the healing rate of peptic ulcer were described either from oral, local or systemic application by many authors (Itoh et al., 1993; Brzozowski et al., 2001), and the potential clinical applications of recombinant EGF generated much interest. However, mentions of EGF in recent articles and reviews on peptic ulcer disease have banished (Yuan, Padol and Hunt, 2006; Chung, Chiang and Lee, 2015; Lanas and Chan, 2017). Partial degradation of EGF in the gastrointestinal tract by action of proteases present in the gastric juice and its consequent impairment of biological activity has been reported (Calnan et al., 2000).

EGF is initially produced as a 1207 amino acid precursor, which is processed into a "mature" 53-amino acid peptide (EGF_{1-53}). In the gastrointestinal tract, EGF is naturally C-terminal truncated by cleavage of proteases present in the gastric juice, being a 49-amino acid, C truncated peptide (EGF_{1-49}) the main partially degraded form in the gastrointestinal tract. EGF_{1-49} shows a decreased biological activity when compared to EGF_{1-53}.

2.3 Metallocarboxipeptidases

The role of proteolytic enzymes is essential for life. By hydrolysing the peptide bond, peptidases participate in the broad spectrum of peptide and protein degradation, as observed during digestion, remodelling and resorption of tissue, and intracellular scavenging of mis-folded proteins and peptides. Proteolysis can also be highly selective as in processes like activation or inactivation of signal molecules, factors, peptide hormones, pro-proteins and enzymes. Because of such an implication, protease activity govern many major processes in life (Gomis-Rüth, 2008).

Carboxypeptidases are exopeptidases that catalyse the hydrolysis of peptide bonds at the C-terminus of proteins and peptides (Vendrell, Querol and Avilés, 2000). Amongst them, metallocarboxypeptidases (MCP) are defined by exerting catalysis by one or two tightly bond divalent metal ions, mostly zinc (Gomis-Rüth, 2008). Metallocarboxypeptidases participate in several processes such as digestion, blood coagulation and fibrinolysis, and prohormone processing (Covaleda et al., 2012).

The activity of metallocarboxypeptidases can be regulated by the action of exogenous carboxipeptidase inhibitors in many organisms. A wide range of organisms display proteic carboxipeptidase inhibitors. Among them, *Nerita Versicolor* Carboxypeptidase Inhibitor (NvCI) has been found to be the strongest inhibitor for the M14A subfamily of MPCs (Esperante et al., 2017).

3 Gaucher disease

Gaucher Disease (GD) is the most prevalent autosomal recessive Lysosomal Storage Disease (LSD) in humans (Santos and Tiscornia, 2017). GD is caused by the accumulation of glucosylceramide (glucocerebrosides) in several organs due to insufficient activity of the enzyme glucosylceramidase beta or glucocerebrosidase (EC 3.2.1.45) (Brady, 2006). Manifestations may include enlarged spleen and liver, liver malfunction, skeletal disorders or bone lesions that may be painful, severe neurological complications, swelling of lymph nodes and (occasionally) adjacent joints, distended abdomen, a brownish tint to the skin, anemia, low blood platelet count, and yellow fatty deposits on the white of the eye (sclera). Persons seriously affected may also be more susceptible to infection. Some forms of Gaucher's disease may be treated with enzyme replacement therapy.

Parkinson disease (PD) is present in 4% of GD1 patients. Even when PD criteria are not met, over 21% of GD1 patients may exhibit at least one parkinsonian finding (Chérin et al., 2010).

3.1 Historical Background

In 1882, Philippe C.E. Gaucher, a French dermatologist, first described GD in his doctorate thesis as “Primary Epithelioma of the Spleen” (*Épithelioma Primitif de la Rate*). In his thesis, Gaucher thoroughly described the clinic, anatomic and histologic hallmarks of GD from a single clinical case (Gaucher, 1882) and by 1920 the name Gaucher Disease was broadly used to describe this pathology (Kahn, 2007).

Unto the beginning of the 1960’s, it was already known that accumulation of excessive amounts of glucocerebrosides in reticuloendothelial cells from spleen, liver and bone narrow occurred in patients with GD (Halliday, N, Deuel, HJ, Tragerman, L, 1940; From and York, 2958; Agranoff, Radin and Suomi, 1962; Brady, Kanfer and Shapiro, 1965), but it wasn’t until 1965 that Roscoe Brady’s group described a notable diminution of the activity of a “glucocerebrosides-cleaving enzyme” in the spleen from patients with GD (Brady, Kanfer and Shapiro, 1965). The molecular basis of GD were reported in the late 1980s as the gene mutations of Glucocerebrosidase were identified (Chen and Wang, 2008).

Although GD was first described in 1882, it wasn’t until 1991 that an effective Enzime Replacement Therapy (ERT) with alglucerase (Cerdase™, Genzyme Corp) became available. ERT revolutionized the treatment of patients with GD because of its ability to revert the disease’s clinical manifestations (Grabowski, 1995). Other therapies specific for GD are Substrate Reduction Therapy (SRT), with several treatments approved by the Food and Drug

Administration (FDA) since the early 2000s and the use of pharmacological chaperones, a novel approach that may be available for GD treatment in a near future (Marcucci et al., 2014).

3.2 Types of Gaucher Disease

Gaucher Disease is traditionally classified 3 different forms according its clinical manifestations: GD type 1, an adult chronic non-neuropatic form (NIM 230800); GD type 2, an infantile, acute neuropathic form (NIM 230900); and GD type 3, a juvenile sub-acute neuropathic form (NIM 321000) (Marcucci et al., 2014).

GD Type 1, the non-neuropathic form, is the most common form in the Western countries with an estimated frequency of 1:40,000-60,000 in the general population (Jung et al., 2016) with an ethnic predilection amongst Ashkenazi Jews, whom present an expected birth frequency around 1:850 (Marcucci et al., 2014). The high prevalence of GD in the Ashkenazi Jews population is suggested by a founder effect originated from many bottlenecks in the Jewish population size since the beginning of the Jewish Diaspora (Chen and Wang, 2008).

GD type 2, the most severe of the clinical manifestations of Gaucher Disease, is a fulminant neuropathic disease, lethal during the first years of infancy, and presents no ethnicity predilection (Marcucci et al., 2014; Chen and Wang, 2008).

GD type 3 also presents neurological involvement but those symptoms tend to appear later in life than in GD type 2 (Jmoudiak and Futerman, 2005). GD type 3 is subdivided into type 3a, 3b and 3c. Type 3a presents predominance of neurological signs over visceral features; type 3b presents the opposite situation, with visceral features over neurological signs; and type 3c is characterized by a rare cardiac variant. GD type 3 is predominant amongst Asian and Arab populations, being type 3c described mainly amongst Palestinian Arabs (Marcucci et al., 2014).

The estimated frequency of Gaucher Disease in the general population is 1:50,000 to 100,000 (Marcucci et al., 2014).

3.3 Molecular and Enzymatic Basis of Gaucher Disease

Gaucher Disease results from mutations in the *GBA1* gene (HGNC:4177), which encodes for the lysosomal enzyme glucosylceramidase beta (GCase). GCase is an hydrolytic enzyme that catalyses the cleavage of glucosylceramide into glucose and ceramide (Horowitz et al., 2016;

Brady, 2006). These mutations result in deficiency and dysfunction of GCase, and a subsequent multisystemic accumulation of glucosylceramide in the lysosomes of macrophages in several organs, mainly the spleen, liver, bone narrow, bone mineral, and, to a lesser extent, in the lungs, skin, heart and kidney (Marcucci et al., 2014).

GCase is a lysosomal membrane associated glycoprotein, which is translated into a 56 kDa polypeptide by endoplasmic reticulum (ER)-bound polyribosomes. The polypeptide is translocated through the ER membrane, its signal sequence cleaved and protein glycosylation occurs on four asparagine residues. Through Golgi apparatus transportation, the high mannose moieties undergo several modifications unto the protein is finally transported to the lysosomes as a 59-63 kDa mature protein. The pathway by which Gcase reaches the lysosome is mannose 6 phosphate receptor-independent (Ron and Horowitz, 2005). It has been reported that misfolded mutants of GCase present variable levels of ER retention and proteasomal degradation. ER retention levels and proteasomal degradation have a direct correlation with GD severity (Marcucci et al., 2014; Ron and Horowitz, 2005).

3.4 Genetics of Gaucher Disease

The *GBA1* gene is located on chromosome 1q21, it is 7.6 kb in length with 11 exons (Schapira, 2015) and encodes for a 536 residue protein, including a 39 residue signal sequence (P04062, Uniprot). The mature 497 residue chain has a calculated molecular mass of 55.6 kDa (ProtParam, ExPASy). Amino acid residue number regarding GBA mutations in this thesis will be displayed according to the traditional residue numbering, which excludes the first 39 amino acids from the signal sequence (Horowitz et al., 2016).

By the time this thesis was last revised, as of May 1st 2018, more than 400 GBA mutations are listed in the Human Gene Mutation database, being more than 200 directly associated with Gaucher Disease (HGMD, Institute of Medical Genetics in Cardiff). Mutations include mainly point mutations, splice site mutations, deletions and recombinant alleles, resulting from recombination between the GBA gene and a 5.5 kb pseudogene (ψGBA) that shares 96% sequence similarity with the GBA gene and is located 16 kb downstream of the functional gene, in the same locus. Point mutations account for more than 80% of GD alleles, whereas insertions, deletion and other complex alleles are responsible for the reminder (Marcucci et al., 2014).

The most prevalent mutant genotype in type I Gaucher disease is the point mutation N370S. This mutation account for around a 75% of the mutant alleles in Jewish patients, and around a 30% in non-Jewish patients. The frame shift mutation 84GG accounts for another 12% of the mutant alleles in Ashkenazi Jews, and five mutations alone account for over 97% of GD alleles in this population. The most prevalent mutation amongst non-Jewish patients is the L444P (Ron and Horowitz, 2005; Chen and Wang, 2008; Beutler, 1995).

Genotype-phenotype correlation in Gaucher Disease is highly inaccurate. GD type 1 can be a symptomatic disease from early childhood, with clinical involvements being visceral manifestations as hepatomegaly, splenomegaly, anaemia and thrombocytopenia, and bone disease, including pain, bone crises and avascular necrosis. However, 90% of N370S homozygous patients are mildly symptomatic or completely asymptomatic (Zuckerman et al., 2007). Many people homozygous for N370S mutation are never diagnosed or detected during genetic screenings.

3.5 Therapeutic Approaches for Gaucher Disease

Before 1991 there were no specific treatments for Gaucher Disease. Total or partial splenectomy was performed in with splenomegaly or pancytopenia as it produced a normalization of platelet counts, decreased fatigue and bleeding, improved abdominal discomfort and increased growth in children. Unfortunately, spleen removal caused progressive liver deterioration and skeleton involvement (Marcucci et al., 2014). Splenectomy nowadays may be performed laparoscopically although is rarely recommended, except for patients unresponsive to other therapies. ERT post-splenectomy is highly advised as protection against skeletal damage and hepatic complications (Zimran, 2011; Marcucci et al., 2014).

Back in 1966, Roscoe O. Brady, a year after elucidating the biochemical basis of GD (Brady, Kanfer and Shapiro, 1965), speculated that if an enzyme was insufficiently active, attempt to purify that enzyme and inject it into patients was a strategy worth to investigate (Brady, 1966, 2006). At the beginning of the 1970s, the first attempt on Enzyme Replacement Therapy in LSD were performed with hexosaminidase A in a patient of the O-variant form of Tay-Sachs disease, with a modest outcome as results showed that none of the injected enzyme had reached the brain (Brady, 2006). The inability to cross the Blood-Brain Barrier (BBB) of injected enzymes represents today one of the main challenges of ERT, as there are no reported improvements on the neurological signs in LSD patients treated with ERT (Brady, 2006; Horowitz et al., 2016).

Back in 1991, however, and after decades of research, the management of GD was revolutionised due to the first approval by the FDA of an Enzyme Replacement Therapy. Alglucerase was a placenta-derived macrophage-targeted glucocerebrosidase (Cerdase, Genzyme Corp). Regular infusions of the enzyme successfully reduced hepatosplenomegaly, improved hypersplenism, anaemia and thrombocytopenia, and ameliorated bone pain in a general regression of the clinical manifestations of GD type 1 (Marcucci et al., 2014; Grabowski, 1995). Alglucerase main limitation was its finite availability, as for each patient about 10 to 12 tons of placentae were required for purification.

In 1994, Imiglucerase (Cerezyme, Genzyme Corp), a recombinant GCase produced in Chinese hamster ovary cells was approved by FDA. Imiglucerase proved to be as effective and safe as Alglucerase but due to its recombinant origin, providing a theoretically unlimited supply and free of pathogenic contaminants, it shortly oust Alglucerase as the treatment of choice for GD type 1 (Grabowski, 1995; Zimran et al., 1995; Marcucci et al., 2014).

Imiglucerase maintained its hegemony as the only ERT for GD until 2009, when Genzyme Corporation announced a viral contamination in his manufacturing site, causing a dramatic reduction in its global supply to 20% and making ERT unavailable for many patients or reducing their doses (Zimran, 2011). By then 2 new ERT for GD were being developed and had already completed phase 3 clinical trials, and the shortage of Imiglucerase lead regulatory authorities to prelicense availability by early access programs (Zimran, 2011; Cox, 2010). One of them, Velaglucerase alfa (VPRIV, Shire) was approved by the FDA in 2010 and the second one, Taliglucerase alfa in 2012 (Marcucci et al., 2014).

Velaglucerase alfa is the only protein amongst those available for ERT in Gaucher Disease whose sequence is identical to that of the wild type GCase, while Imiglucerase and Taliglucerase alfa have an amino substitution histidine to arginine at position 495 (Marcucci et al., 2014). This particularity of Velaglucerase appears to be an advantage as a booster effect occurs in some patients switching from long-term treatments with Imiglucerase to Velaglucerase. Moreover, Velaglucerase alfa appears to be associated with fewer hypersensitivity reactions and antibody development than the others ERTs (Elstein et al., 2012; Marcucci et al., 2014). Although it has been reported that Velaglucerase alfa is unable to cross the BBB and thus act on the Central Nervous System (CNS), the drug has been successfully used in neuropathic cases of GD with visceral manifestations that had developed adverse effects to other ERTs (Vairo et al., 2013).

The variable pattern and severity of GD type 1 as well as its uncertain manner of progression complicate the decisions to be made in GD treatment, regarding its implementation and evaluation of the therapeutic response. Evaluation and decision-making must be tailored to the individual patient, but a framework to individualized management was at some point required. In 2004, a panel of experts in GD, after an extensive review of the back then state of the art, established the goals of treatment in GD and proposed a schedule of monitoring of relevant aspects to corroborate the achievement, maintenance and continuity of the therapeutic response in GD patients. Therapeutic goals were set for the main visceral manifestations, them being anaemia, thrombocytopenia, hepatomegaly, splenomegaly, skeletal pathology, pulmonary involvement and growth in paediatric patients (Weinreb, 2007).

3.6 Velaglucerase, a thorough glance

As it has already been mentioned, the introduction of Enzyme Replacement Therapy in GD treatment revolutionised the management of patients enduring this LSD. Not only was it the first therapeutic approach after decades of research that specifically addressed GD but also reached a normalization of many of the GD clinical manifestations (but not the neuropathic manifestations) with a reliable safety profile (Zimran, 2011; Marcucci et al., 2014). Amongst the 3 different ERTs currently available, Imiglucerase, Velaglucerase Alfa and Taliglucerase alfa; Velaglucerase Alfa seems to stand out due to its safer profile and an apparent advantage towards the other to ERTs regarding its wild type sequence.

The first appearance of Velaglucerase Alfa in the bibliography dates back to 2007, in an early study carried out by Doctor Ari Zimran and his team designed to analyse the pharmacokinetics of the, by then, Gene-Activated human Glucocerebrosidase (GA-GCB) (Zimran et al., 2007). Different doses of the drug were tested and the main pharmacokinetic variables were determined. Interestingly, Velaglucerase is rapidly cleared from circulation following first-order elimination kinetics in patients receiving Intravenous (IV) infusions and mean elimination half-life ($t_{1/2}$) was around 10 minutes when 60 U/kg were administrated (ranging from 4 to 15 minutes). The mean residence time (MRT) for the same dose was determined to be 14 minutes. Coherently to these fast elimination kinetics, for 1 hour infusions, time to maximum serum concentration (T_{max}) was 60-65 minutes (Zimran et al., 2007).

Subsequent studies have been performed in order to benchmark the impact of Velaglucerase to meet the therapeutic goals in the treatment of Gaucher Disease, and compare the results from those of Imiglucerase. Mainly anaemia, thrombocytopenia, hepatomegaly, splenomegaly

and skeletal pathology were the analysed clinical parameters for therapeutic assessment. Data undoubtedly indicates Velaglucerase capacity to achieve the therapeutic goals in GD treatment in most of the patients for all the clinical parameters studied (Zimran et al., 2010; Elstein et al., 2011; Morris, 2012; Turkia et al., 2013; Elstein et al., 2012). Velaglucerase results respect Imiglucerase also showed a low rate of hypersensitivity reactions against Velaglucerase treatments and minimal seroconversion (antibody formation against Velaglucerase was around 1%, while 23% of the patients develop antibodies against Imiglucerase) (Elstein et al., 2012; Morris, 2012).

Aside from clinic studies, a complete characterization of Velaglucerase considering its crystal structure, glycosylation and cellular internalization was reported in 2010 (Brumshtein et al., 2010). The solving of Velaglucerase structure allows provides valuable information not only about domains and residue distribution of the protein, but also a detailed reconstruction useful for the active site characterization, the analysis of the protein surface and of the bonding interaction (figure 1.3).

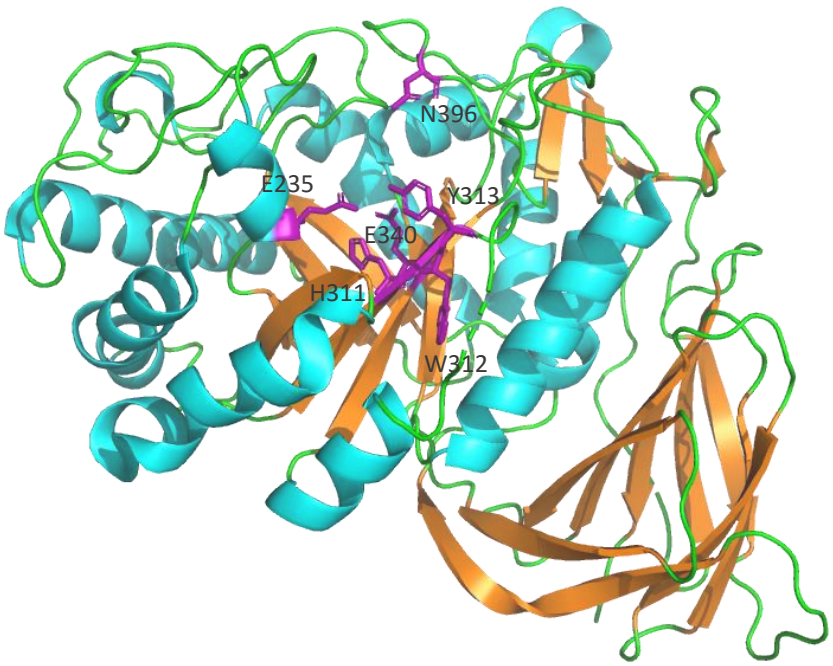

Figure 1.3. Representative structure of Velaglucerase alfa. Ribbon representation of the three-dimensional structure of Velaglucerase alfa (PDB 2WKL). The α-helices are shown in cyan, the β-strands in orange and the loops in green. The side chain of the residues from the active site (E235, H311, W312, Y313, E340 and N396) are depicted in magenta sticks. Image generated with PyMOL 2.1.1 (Schrodinger LLC).

3.7 Future prospects for Gaucher Disease

Although the improvement experienced in Gaucher Disease management in the last decades, especially in the treatment of GD type 1, new directions in the treatment of Gaucher disease are being explored.

Substrate Reduction Therapy (SRT) focuses in a different target than ERT, as instead of removing the accumulation of storage material within the lysosomes, SRT targets the biosynthetic pathway of the substrate to partially reduce its influx. Miglustat (Zavesca, Actelion Ltd) is already approved for oral administration in the treatment of mild-to-moderate GD type 1. However, the effect of SRT on neurologic manifestations of GD has not been stablished yet (Horowitz et al., 2016).

The use of pharmacological chaperones (PCs), small molecules that bind to misfolded proteins and facilitate their refolding, is a promising approach for GD because PCs can be administrated orally, they can penetrate the BBB and their cost is much affordable than ERT. PCs have been described to be able to bind misfolded GCase in the neutral pH of the ER and induce a conformational change of the enzyme. Refolded protein transportation to the Golgi is enhanced and from there it can reach the lysosomes, where the acidic pH produces a dissociation of the PC-GCase complex. A large number of publications have studied different PCs that facilitate the exit of misfolded GCase from the lysosome and increase its activity in the lysosomes. Cinical trials on Ambroxol as a PCs for Gaucher Disease are being carried out (Horowitz et al., 2016).

Studies in overcoming the BBB for drug delivery have been object of study in the last years and encapsulation of drugs is one of those approaches (Saraiva et al., 2016). Encapsulation of proteins used for ERT as a way to overcome their shortcomings in the GD treatment have been timidly addressed, although it has been reported that encapsulation of Velaglucerase Alfa in liposomes may increase its lysosomal delivery into Gaucher cells (Marianecci et al., 2017). Future studies in Velaglucerase alfa encapsulation and further modifications intended to overcome the BBB could open the door to address the neurological manifestations of GD.

4 Parkinson's Disease

Parkinson's Disease (PD) is the second most common neurodegenerative disorder only after Alzheimer's, affecting more than 7 million patients worldwide (Migdalska-Richards and Schapira, 2016). PD is a multifactorial progressive neurodegenerative being the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta its central pathological feature (Blanz and Saftig, 2016). PD's motor symptoms – resting tremor, rigidity and bradykinesia are known as the traditional PD triad, but non-motor symptoms such as depression, constipation, pain, sleep disorders, genitourinary problems, cognitive decline and olfactory dysfunction are gaining recognition (Barkhuizen, Anderson and Grobler, 2016).

Parkinson's Disease was first described as a neurological syndrome in 1817 by the English surgeon James Parkinson (Parkinson James, 1817). Advances in the study of PD regarding demographics, clinical, pathological and morphological details were made astride between the nineteenth and the twentieth century. Worldwide incidence of PD is estimated to be between 5 and 35 new cases per 100,000 individuals every year(Poewe et al., 2017). PD disease is rare before 50 years of age, but its incidence sharply increases 5 to 10-fold from 60 to 90 years old, affecting 1-2% of individuals over 65 years old and rising to 4% over 85 years old (Poewe et al., 2017; Sardi, Cheng and Shihabuddin, 2015). Two of the main characteristics of PD are neuronal loss in specific areas of the substantia nigra and intracellular accumulation of α-synuclein (Poewe et al., 2017).

4.1 α-synuclein proteostasis and degradation

α-synuclein, as being one of the main components of intraneuronal protein aggregates and the fact that its gene, SNCA, can cause heritable forms of PD because of point mutations and multiplications, is without doubt a key part in PD. α-synuclein normal neuronal function occurs in the cytosol and possibly in the mitochondria and the nucleus, but as of today is still not fully understood. During the pathogenic process α-synuclein acquires neurotoxicity by oligomerization at first and subsequently forming small protofibrils that finally progress in large, insoluble fibrils. The decline in proteolytic mechanisms due to ageing might play an important role in α-synuclein accumulation (Poewe et al., 2017).

In normal cells, α-synuclein is degraded by means of the ubiquitin-proteasome system and the lysosomal autophagy system (LAS) in order to maintain intracellular homeostasis. The importance of LAS seems to lay in its primary role in the clearance of α-synuclein oligomers

through both chaperone-mediated autophagy and macroautophagy. Impairment of these degradation systems may contribute to α-synuclein accumulation. Moreover, α-synuclein oligomers have been reported to inhibit the ubiquitin-proteasome system, accumulated α-synuclein can inhibit macroautophagy and different forms of α-synuclein can reduce CMA. Thus, disrupted homeostasis can initiate α-synuclein accumulation able to disrupt its degradation, entering a vicious cycle (Poewe et al., 2017).

4.2 Lysosomes and autophagy

Lysosomes are considered the end-point of the endocytic and autophagic pathways, mainly because more than 60 acid hydrolases are required there for the degradation of proteins, lipids, sugars and other macromolecules (Blanz and Saftig, 2016). Autophagy in particular is a lysosomal pathway involved in the turnover of intracellular proteins and organelles, as well as in the degradation of damaged organelles and the removal of misfolded or aggregated proteins. Autophagy involved in the continuous turnover of proteins and organelles directed towards cellular homeostasis maintaining is known as constitutive autophagy, while increased protein degradation due to change of the extracellular environment is known as inducible autophagy (Stoka, Turk and Turk, 2016; Blanz and Saftig, 2016).

Three different types of autophagy have been described in mammalian cells: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy is the complete sequestration of complete portions of cytosol into a double membrane vesicle or autophagosome, which can entrap not only proteins but also complete organelles. Autophagosomes fuse with lysosomes, acquiring hydrolytic enzymes and acidifying its lumen, and thus favouring rapid degradation. Micro autophagy also consists in the entrapping and degradation of complete regions of the cytosol, differentiating from macroautophagy as the lysosome membrane acts as the engulfing membrane. Chaperone-mediated autophagy consists in specific protein degradation mainly during nutrient deprivation. While no intermediate vesicles are needed, a motif in the aminoacid sequence of the target proteins is required. Substrate proteins are translocated from the cytosol through the lysosomal membrane directly into the lumen (Cuervo, 2004). All pathways converge at the level of lysosomes, where degradation of the different organelles and biomolecules take place.

4.3 GBA involvement in Parkinson's Disease

Parkinsons Disease aetiology remains elusive. Most of the cases occur in an aparent sporadic or idiopathic manner, and only around 10% of PD cases are inheritable and attributable to mutation in specific genetic loci (Chai and Lim, 2014).

As it already has been described, the human GBA1 gene encodes for the glucocerebrosidase, a hydrolytic, lysosomal enzyme that catalyses the cleavage of glucosylceramide into glucose and ceramide. In homozygosis, mutations in this gene are responsible for Gaucher Disease. However, the fact that mutations in this gene is, to date, the most important genetic predisposing risk factor for PD was not anticipated nor expected (Sardi, Cheng and Shihabuddin, 2015). Findings in clinic studies suggested that there was an increased occurrence of Parkinsonism not only in GD patients, but also in their carrier family members. According to the HGMD, among the more than 400 mutations described for GBA, around 20-30 are directly related with PD. Mutations N370S and L444P are the most common in both, GD and PD (Schapira et al., 2016).

It has been estimated that GBA1 mutations confer a 20 to 30-fold increased risk in developing PD, with some studies showing a penetrance for PD development of 20% at 70 years old and 30% at 80 years old, either in homozygous and heterozygous. 7 to 10% of PD have a GBA1 mutations, meaning that numerically GBA1 mutations are the most important risk factor for PD. PD associated with GBA1 mutations (PD-GBA1) is clinically indistinguishable from idiopathic PD, but it does show a slightly earlier onset and a tendency to more cognitive impairment (Schapira et al., 2016; Migdalska-Richards and Schapira, 2016).

Lysosomal dysfunction in LSD such as GD causes a progressive accumulation of glucosylceramide. Sphingolipids accumulation alters autophagy by reducing autophagosome clearance and inducing cell death. However, it seems unlikely that GCase role in PD is autophagy disruption by sphingolipids accumulation, as it requires homozygosis to cause sufficient accumulation to reach a pathogenic phenotype, and PD-GBA1 shows no difference between homozygous and heterozygous individuals (Migdalska-Richards and Schapira, 2016). Several studies have been carried out investigating the levels of GCase and GCase activity in the brain of PD patients, both idiopathic or PD-GBA1. In heterozygous PD-GBA1 patients, a significant decrease in GCase activity was observed in practically all brain areas, being the substantia nigra the most affected area followed by the cerebellum. Interestingly, GCase activity was also reduced in the substantia nigra and cerebellum of idiopathic PD patients, who carried no mutations for GBA. Decrease in GBA activity in PD patients carrying no mutations

indicates a possible contribution of GCase to the pathogenesis of PD, although the origin of GCase deficiency in idiopathic PD is unclear (Schapira et al., 2016). Relationship between GCase and α-synuclein seems to be reciprocal, nonetheless. Inhibition of GCase activity leads to accumulation of α-synuclein and overexpression of α-synuclein reduces GCase activity. But also, α-synuclein knockouts seem to increase its GBA activity, and an increase of GBA activity levels reduces α-synuclein levels (Schapira et al., 2016).

Objectives

OBJECTIVES

The main objective of this thesis was to improve the therapeutic and pharmacological properties of very specific types of proteins with clinical applications, by means of different nanotechnological approaches. The methodologies included from the encapsulation of the proteins (NvCI, EGF) with PLGA to the conjugation of an enzyme (GBA) with a polypeptide coating. The goal is to increase the stability of the proteins and improve their therapeutic efficacy and efficiency.

Specific objectives for the different approaches were:

Design of a therapy based in the encapsulation of EGF for treatment of Peptic Ulcers.

- To design, synthesize and characterize the encapsulation of NvCI and EGF proteins with Poly(lactic-*co*-glycolic acid) (PLGA), a biocompatible polymer approved by the regulatory agencies FDA and EMA.
- To co-encapsulate a mixture of two proteins (NvCI and EGF) in a single nanocapsule, maintaining their respective activity: EGF with mitogenic activity and NvCI with protease inhibitory activity.
- To optimize the encapsulation conditions in order to modulate the characteristics of the resultant nanoparticles, including size, monodispersion, surface charge, stability in biological media and protein encapsulation efficiency.
- To evaluate with different *in vitro* assays the activity of the proteins and their therapeutic value.
- To optimize the parameters of the encapsulation process for increasing the encapsulation efficiency, the stability of the proteins and the therapeutic activity.

Improvement of the Enzyme Replacement Therapy for Gaucher Disease treatment

- To evaluate different approaches (encapsulation/conjugation) based in biocompatible polymers for the stabilization of Velaglucerase alfa and increase its therapeutic action
- To synthesize and characterize Velaglucerase conjugated with Poly(L-glutamic acid) (PGA) using the Polymer Unmasked-Masked Protein Therapy (PUMPT) methodolgy, maintaining the enzymatic activity.
- To optimize the experimental parameters for the conjugation process and minimize the decreasing of enzymatic activity during the conjugation process.
- To evaluate the therapeutic efficiency of Velaglucerase conjugates in an *in vitro* cellular model for lysosomal dysfunction.
- To evaluate the stability of Velaglucerase conjugates *versus* the unconjugated enzyme when exposed to human blood plasma.

Chapter II

Experimental procedures

CHAPTER II: EXPERIMENTAL PROCEDURES

1 Protein quantification

1.1 BCA Assay

Bicinchoninic Acid (BCA) assay is a detergent-compatible technique for the colorimetric detection and quantification of total protein with high sensitivity and low protein-to-protein variation (Smith et al., 1985). Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used for total protein quantification when possible, following the Microplate Procedure detailed in the manufacturer´s protocol. Bovine Serum Albumin (BSA) standards were freshly prepared from 2 mg/ml BSA stocks (Thermo Scientific) according to the enhanced protocol, using the buffer from the samples as a diluent.

BCA assay following the enhanced protocol is sensitive for protein ranges from 5 μg/ml to 250 μg/ml. Samples liable to exceed the assay maximum needed to be diluted into range using the buffer from the samples as a diluent.

25 μl of standards and samples were added in triplicates in a 96-well microplate. 200 μl of the Working Reagent from the Assay kit were added per well and the microplate was incubated for 30 minutes at 37°C.

Absorbance was subsequently measured on a Victor^3 1420-041 Multilabel Plate Reader (Perkin Elmer), at 550 nm.

1.2 Bradford Assay

Bradford assay is a total protein determination method useful for its reproducibility, stability and rapidity (Bradford, 1976). Coomassie (Bradford) Protein Assay Kit (Thermo Scientific) was used for total protein quantification when possible, following the Microplate detailed in the manufacturer´s protocol. Bovine Serum Albumin (BSA) standards were freshly prepared from 0.1 mg/ml BSA stocks (Thermo Scientific) using the buffer from the samples as a diluent.

150 μl of the standards and samples were added in triplicates in a 96-well microplate. 150 μl of the Assay Kit reagent were added per well. Absorbance was subsequently measured on a Victor^3 1420-041 Multilabel Plate Reader, at 620 nm.

1.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a widespread technique based on the separation of proteins in a polyacrylamide gel when an electric field is applied. Many factors intervene in protein mobility in a Polyacrylamide Gel when an electric field is applied:

- Protein size: the bigger they are, the slower they migrate.
- Charge type: positively and negatively charged protein migrate in opposite directions.
- Charge density: the higher the net charge per mass unit, the faster they migrate.
- Protein morphology: protein conformation (globular, lineal, unstructured…) affect protein mobility in a fluid.

In order to use electrophoresis for protein separation by size, protein charge, charge density and protein morphology must be equalised in all the proteins in the sample so that protein size is the only variable affecting protein migration. Sodium dodecyl sulphate (SDS) is an anionic surfactant with denaturing properties for proteins. At high concentrations, SDS binds to protein with high affinity, breaking the weak bonds involved in the secondary and tertiary structure of proteins and forcing proteins into a rod-like conformation by electrostatic repulsion. SDS charge shields the original charge of proteins and provides a net negative charge, with a charge-to-mass ratio essentially identical for all proteins.

Covalent bonds involved in protein conformation, basically disulphide bonds between cysteine pairs, need to be reduced by incubation with β-mercaptoethanol as SDS is unable to break them by itself. Disulphide bonds reduction and SDS denaturalization combined successfully denature proteins into a rod-like shape.

With charge type, charge density and protein morphology homogenised, protein size is effectively the only variable influencing protein migration in a homogenous polyacrylamide gel under a constant electric field. By using these properties, SDS-PAGE is used for size-dependant protein separation.

Conventional SDS-PAGE was performed using the standard protocol (Laemmli, 1970) detailed below. Buffer conditions are detailed in **Table 2.1.**

Table 2.1. Polyacrylamide gels composition

Chemical	Separating	Stacking
Tris-HCl (Sigma)	375 mM (pH 8.8)	125 mM (pH 6.8)
SDS (Sigma)	0.1% w/v	0.1% w/v
Ammonium Persulfate (Sigma)	0.05% w/v	0.05% w/v
Acrylamide (NBS Biological)	8-20% v/v (variable)	4% v/v
TEMED (GE Healthcare)	0.15% v/v	0.025% v/v

Samples were quantified for total protein and diluted as each case required. Samples were mixed with 0.2 volumes of Laemmli buffer 6x (2% w/v SDS, 10% v/v Glycerol, 60 mM Tris-HCl pH 6.8, 0.01% v/v Bromophenol Blue and 2% v/v β-mercaptoethanol), incubated for 5 minutes at 95ºC and loaded into the gel. Samples were then run in an electrophoresis chamber with 1X Running Buffer (25 mM Tris-HCl, 192 mM Glycine and 0.1/SDS) for 15 minutes at a constant voltage of 90 V for protein stacking and variable time at 190 V for protein separation (as required).

SDS-PAGE protein separation is normally coupled with protein identification techniques. Protein staining techniques such as silver staining can be used for unspecific protein detection in a polyacrylamide gel. The use of western blot techniques after SDS-PAGE is used for specific protein detection.

1.4 Western blot

Western blot is an immunochemical technique based on the ability of antibodies to specifically attach to an antigen, epitopes of proteins in that particular case. Because of the high affinity of antibodies towards their specific antigen, immunochemical techniques can be used to detect and quantify specific proteins.

Immunochemical detection can be used with other techniques. Western blot is the immunochemical detection of specific proteins after separation with polyacrylamide gel electrophoresis. Because of the high density of acrylamide and the large size of antibodies, antibodies are unable to penetrate polyacrylamide gels. Proteins are transferred from the electrophoretic gels into membranes with high affinity for proteins (nitrocellulose or

polyvinylidene difluoride, PVDF). Protein transfer maintain the distribution of proteins after SDS-PAGE and allow antibodies to interact with them (Eisenstein, 2005).

After SDS-PAGE, separated protein samples were transferred onto Polyvinylidene Fluoride (PVDF) membranes (Immobilon-P, Millipore). Target proteins were detected using primary antibodies, listed in table 2.3. Protein bands were developed with chemiluminescence (Westar ηC, Cyanagen) after incubation with HRP-labelled secondary antibodies: either goat anti-mouse (1:5000, BioRad) or donkey antirabbit (1:5000, GE Healthcare). Images were taken using a Molecular Imager Versadoc MP4000 system (Biorad).

Transfers were run in an electrophoresis chamber with 1X Transfer Buffer (25 mM Tris-HCl, 192 mM Glycine and 10% Methanol) at a constant voltage of 100V (perpendicular to the gel). Transfer time varied according to the target protein, as indicated in table 2.2.

After transfer, membranes were reversibly stained with a Ponceau S solution to assess that the transfer was successfully completed. Ponceau S solution was removed by three washes of 5 minutes with TBST buffer (Tris Buffered Saline – Tween, Tris Base 19 mM, NaCl 137 mM, KCl 2,7 mM, 0.1% v/v Tween 20), under mild shaking. In order to avoid unspecific union of the antibodies in the membrane due to PVDF high affinity for proteins, the membrane was blocked through an incubation with a 5% solution of non-fat dry milk (Central Lechera Asturiana) in TBST for 30 minutes at RT under mild shaking.

The blocking solution was removed and the membrane was washed 3 times with TBST for 5 minutes under mild shaking, at room temperature. The primary antibody, diluted in a 2% solution of non-fat dry milk in TBST in the concentration indicated in the Table 2.3, was incubated for 90 minutes with the membrane under mild shaking, at RT. The primary antibody was then recovered and the membrane washed 3 times with TBST for 5 minutes, under mild shaking. A secondary antibody targeted towards the primary antibody was freshly diluted 1:5000 in a 2% solution of non-fat dry milk in TBST and incubated with the membrane for 30 minutes. Membranes were washed 3 times with TBST for 5 minutes, under mild shaking and developed afterwards.

Table 2.2. Protein antigens detected by Western Blot and their transfer time

Antigen	Molecular weight (kDa)	Transfer time (min)
EGF	6.2	45
NvCI	5.9	45
EGFR	134	240
GBA	59.7	90
GADPH	36	240

Table 2.3. Primary antibodies for Western Blot

Antibody	Reference	Antigen	Type	Dilution	Provider
Anti-GBA	ab55080	Human GBA	Mouse monoclonal	1:1000	Abcam
Anti-EGFR	Sc-71032	Human EGFR	Mouse monoclonal	1:1000	Santa Cruz
Anti-pEGFR	Sc-101668	Human pEGFR (Y1173)	Rabbit polyclonal	1:2000	Santa Cruz
Anti-NvCI	N/A	NvCI	Rabbit antiserum	1:500	ProteoGenix
Anti-EGF, clone 3A8	M806	Recombinant human EGF	Mouse monoclonal	1:2500	Invitrogen
Anti-GADPH	AM300	Rabbit GADPH	Mouse Monoclonal	1:1000	Ambion

2 Heterologous Expression and Purification of Recombinant NvCI and EGF

Nerita versicolor Carboxipeptidase Inhibitor (NvCI) and recombinant human Epithelial Growth Factor (rhEGF) were similarly expressed in the *Pichia pastoris* expression system (GeneArt) as described in previous publications (Covaleda et al., 2012). The fermentation supernatant was adjusted with citric acid (Sigma) and sodium hydroxide (Sigma) to a citrate concentration of 0.1 M and pH 3.2, centrifuged at 6000 rpm at 4ºC for 20 minutes, decanted and filtered on a 0.22 µm Polyethersulfone (PES) filter (Millipore).

NvCI and rhEGF purification was performed using a combination of a cation exchange chromatography and a size exclusion chromatography. An ÄKTApurifier system (GE Healthcare) was used in both cases, using a XK 50/30 Column (GE Healthcare) packaged with Streamline Direct HST Resin (GE Healthcare) for the cation exchange chromatography and a Superdex 30 High Load Column (GE Healthcare) for the size exclusion chromatography.

The buffered fermentation supernatant was loaded into the Streamline Direct HST Resin-packaged XK 50/60 Column duly equilibrated with citrate buffer 0.1 M pH 3.2, and the unbound washed with 2 column volumes (CV) of the same citrate buffer. Elution was performed using a linear gradient from 0% to 100% of phosphate buffer 0.1 M, pH 8.0 for 3 CV and collected in 10-millitre fractions. Protein elution was monitored by absorbance at 214 nm and 280 nm as well as by MALDI-TOF-MS. NvCI or EGF-containing fractions were concentrated using a 3 kDa MWCO Amicon Ultra 15 ml filters for protein purification and concentration (Merck Millipore) and their buffer changed to PBS pH 7.4.

Concentrated fractions from the cation exchange chromatography were further loaded into a Superdex 30 High Load column, duly equilibrated with PBS pH 7.4. An isocratic elution with PBS pH 7.4 (flow rate of 2.5 ml/min) was performed and 5-millilitre fractions were collected. Protein elution was monitored by absorbance at 214 nm and 280 nm as well as by MALDI-TOF-MS. NvCI or EGF-containing fractions were concentrated using a 3 kDa MWCO Amicon Ultra 15 ml filters for protein purification and concentration (Merck Millipore).

The purity of NvCI and EGF was determined by MALDI-TOF-MS (Covaleda et al., 2012) and final protein concentration was ascertained by BCA quantification.

3 Protein encapsulation in PLGA nanoparticles

3.1 Synthesis of PLGA nanoparticles

PLGA nanoparticles were synthesised using a solvent emulsification-evaporation method based on a water in oil in water (w/o/w) double emulsion technique, a modified version of a previous published method (Fonte et al. 2014).

25 mg of Resomer RG 503 H, Poly(D,L-lactide-co-glycolide) (Sigma) were dissolved in 750 µl of dichloromethane (Scharlau) under magnetic stirring. 30 µl of Span 80 (4% v/v, Sigma) were added as a surfactant for the oil phase in order to prevent the merging of droplets, with critical effects on size and encapsulation efficiency (Taghipour et al., 2014). The inner face, aqueous, was a solution of the peptide or protein to encapsulate (peptide concentration and buffer composition varied as necessary, as indicated in table 2.4). 75 µl of peptide solution was added to the PLGA solution and mixed with a Branson Sonifier SFX550 (Emerson Electric Co.) using a microtip for 90 seconds with 25% of amplitude. After homogenization, the obtained emulsion was poured onto 9.4 ml of 2% w/v PVA (Sigma), 1 M NaCl and mixed under the same conditions of sonication. This secondary emulsion was subjected to magnetic stirring until complete evaporation of the dichloromethane. The resultant nanoparticles were washed three times with MilliQ water by centrifugation at 30000 g for 45 minutes at 4°C and finally redispersed in 1 ml of water. Nanoparticles were used right away or freeze-dried for long term storage.

Table 2.4. Composition of the aqueous inner phase (W_1) of PLGA nanoparticles-

Protein	[Protein] (mg/ml)	Buffer
Lysozyme	2.5	PBS
Velaglucerase alfa	2.5	VCB
NvCI	3	PBS
EGF	3	PBS

3.2 Nanoparticles Size and Zeta Potential measurement

Nanoparticle suspensions were diluted in MilliQ water, PBS buffer pH 7.4 and PBS buffer with 0.5 mM BSA to 0.007% w/v for particle size measurement. Suspensions diluted in MilliQ water

were also used for zeta potential assessment. A Zetasizer Nano ZS (Malvern) was used for both measurements: the particle size was assessed by dynamic light scattering and the zeta potential by phase analysis light scattering. All measurements were performed in triplicate.

3.3 Scanning Electron Microscopy

The shape and the surface morphology of the PLGA nanoparticles was observed using Scanning Electron Microscopy (SEM). Aluminium stubs (Ted Pella) were coated with SEM conductive aluminium tape and 3 10-microlitre droplets from each nanoparticle solution (0.007% w/v) were deposited and dehydrated in a desiccator. Samples were vacuum-coated with a 5-nm layer of platinum in a High Vacuum Sputter Coater EM ACE600 (Leica) and observed on a Quanta 650 FEG scanning electron microscope. The measurements were carried out at 20 kV (FEI, Thermo Scientific).

3.4 Freeze-Drying for long-term storage of Nanoparticles

The synthesised PLGA nanoparticles dispersed in MilliQ water were aliquoted into tared 1.5-millilitre Eppendorf tubes. 4% w/v of sucrose could be added as a cryoprotectant. Tubes were sealed with Parafilm (Bemis NA), pierced several times with a needle and placed into a freeze drying flask. Nanoparticles were frozen at -80°C for 2 hours and transferred to a LyoQuest -85 Freeze Dryer (Telstar) at 0.035 mbar for 48 hours at -80°C.

Reconstitution of freeze-dried nanoparticles was done by adding MilliQ water into the Eppendorf tubes. Nanoparticles were then sonicated in an Elmasonic S30H Ultrasonic Cleaner (Elma) for 10 minutes.

3.5 Characterization of PLGA nanoparticles

Protein Encapsulation Efficiency

The protein encapsulation efficiency (EE) was indirectly determined based on the quantification of non-encapsulated protein. The amount of encapsulated protein was calculated

$$EE = \frac{\text{total amount of protein} - \text{free protein in the supernatants}}{\text{total amount of protein}} x100$$

(1)

The free protein in the supernatant, either in the case of NvCI or rhEGF, is the amount of protein that remained in the aqueous phase after centrifugation at 30000 g for 45 minutes at 4°C.

Specific protein quantification was performed by RP-HPLC. Supernatant samples were acidified with 0.1% v/v aqueous TFA and loaded into a Nova-Pak C8 cartridge (Waters), 4 µm particle size, 3.9x150mm (Waters) equipped in a 2695 Separation Module (Waters) and analysed using a multistep gradient elution. Solution A was 0.1% v/v of TFA in MilliQ water and Solution B was 0.1% v/v of TFA in Acetonitrile (ACN). Elution started at 5% of ACN followed by a 30-minute linear gradient from 5% to 35% ACN. Next, ACN raised to 98% in a 5-minute lapse, and was 98% ACN was maintained for 5 more minutes. ACN levels were then dropped again to 5% in a one-minute gradient and 5% ACN was maintained for a final 9 minutes. Protein elution was monitored by absorbance at 214 nm and 280 nm using a 2487 Dual λ Absorbance Detector (Waters). Samples were run in triplicate.

4 Polymer Masked-Unmasked Protein Therapy

Polymer Masked-Unmasked Protein Therapy (PUMPT) was presented by María J. Vicent and her group around ten years ago (Duncan et al., 2008). This technique, designed to be used in protein-based therapeutics consists in conjugating a protein to a biodegradable polymer, thus increasing its stability in the bloodstream and masking its activity during transport. By polymer degradation in the site of action of the conjugated protein, its activity was unmasked (Talelli and Vicent, 2014).

Recently, a variation of the PUMPT concept was presented also by Vicent and her group ((Talelli and Vicent, 2014), introducing the use of a reduction sensitive linker in conjugation, so protein unmasking and release relies on the cleavage of that sensitive linker in specific sites of the body. This variation also introduced the use of Poly(L-glutamic acid) (PGA), a nonimmunogenic, nontoxic and biodegradable polymer; many conjugates with PGA have reached clinical trials.

4.1 Materials

Velaglucerase alfa (VPRIV™) is a recombinant form of human β-glucocerebrosidase produced by Shire Human Genetic Therapies, Inc. (Zimran et al., 2007). A 400 Unit vial containing 10 mg of VPRIV as a freeze-dried powder was received and resuspended in 4 ml of sterile, MilliQ water. 500 µl aliquots were made, frozen in liquid nitrogen and stored at -80ºC. VPRIV sequence is identical to the natural human protein (P04062, Uniprot). Poly(L-glutamic acid) (PGA, acid form; Mn = 30500 Da) was supplied by Polypeptide Therapeutic Solutions SL (PTS, Spain). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium (DMTMM) chloride was synthesized in Polymer Therapeutics Lab, Centro de Investigación Principe Felipe (CIPF, Spain), obtained essentially as described in Kunishima et al. (1999). Pyridyl dithiol ethylamine HCl salt was synthesized in Polymer Therapeutics Lab, Centro de Investigación Principe Felipe (CIPF, Spain), synthesised essentially as described in van der Vlies et al. (2010).

4.2 Synthesis of Pyridyl Dithiol modified PGA (PGA-PD)

Pyridyl Dithiol modified PGA was synthesised essentially as described in Talelli and Vicent (2014). PGA was dissolved in MilliQ water at 0.5 Mm and sufficient sodium bicarbonate

(Sigma) was added under magnetic stirring to ensure complete dissolution of the PGA (by converting the glutamic acid residues to their sodium salt form). Then, 0.3 molar equivalents of DMTMM chloride were added still under magnetic stirring, for 15 minutes (molar equivalents are calculated from a single glutamate residue instead of the whole polymer). After DMTMM activation of carboxyl groups, 0.15 molar equivalents of pyridyl dithiol were added and Ph was adjusted to 8 with sodium bicarbonate. The reaction was stirred overnight and the resultant PGA-PD was washed by centrifugation with a 3 kDa MWCO Amicon Ultra 4 ml (Merck Millipore). The purified polymer was frozen with liquid nitrogen and then freeze dried.

The resulting product was analysed by NMR to calculate the percentage derivatization of PGA with pyridyl dithiol groups, and from this the molecular weight of the modified polymer was also calculated. The principal NMR signals analysed were: ^{1}H NMR (300 ppm, D2O): 8.4 ppm (m, CH_{aromat}), 7.84 ppm (m, CH_{aromat}), 7.28 ppm (m, CH_{aromat}), 4.33 ppm (m, NH-CH-CO of PGA main chain), 3.48 and 2.95 ppm (m, CH2CH2 of ethylamine), 1.9−2.3 ppm (m, CHCH2CH2COOH of PGA side chain protons). The molecular weight of the modified polymer was 151 for the GA and 297 for the pyridyl dithiol GA units.

4.3 Modification of VPRIV with N-Succinimidyl-S-acetylthiopropionate (SATP).

Velaglucerase was modified with N-Succinimidyl-S-acetylthiopropionate (SATP, Thermo Scientific) essentially as described in the User Guide: SATA and SATP supplied by the manufacturer. Velaglucerase buffer (VCB, 44 mM sodium citrate, 6 mM citric acid, 0.01% v/v Tween20) was replaced for PBS at pH 7.4 with 0.01% v/v of Tween 20, in a concentration of 0.5 mg/ml. SATP was dissolved with DMSO to a 20 mg/ml concentration and Velaglucerase was incubated with 10 molar equivalents of SATP for 45 minutes at room temperature under mild shaking (100 rpm). The resultant SATP modified Velaglucerase (GBA-SATP) was washed three times with VCB using a 3 kDa MWCO Amicon Ultra 0.5 ml (Merck Millipore) (Talelli and Vicent, 2014).

4.4 Conjugation of GBA-SATP to PGA-PD

In order to conjugate GBA-SATP to PGA-PD, one volume of GBA-SATP was mixed with 0.1 volumes of PGA-PD in VCB, containing 5 molar equivalents of PGA-PD. As the sulfhydryl groups from the SATP modification need to be unprotected, 0.1 volumes of deacetylation solution (User Guide: SATA and SATP, Thermo Scientific) were added, and the reaction was incubated

at room temperature for 2 hours. The final product, GBA-PGA was washed using a 30 kDa MWCO Amicon Ultra 0.5 ml (Merck Millipore). The purified product was frozen with liquid nitrogen and freeze dried (Talelli and Vicent, 2014).

4.5 Glutathione mediated release

In order to study the release of Velaglucerase from the conjugates under the reducing conditions found in intracellular environments, conjugates were incubated for one hour at 37°C in the presence of 10 mM of reduced glutathione (GSH) under nitrogen atmosphere (to avoid oxidation of glutathione by oxygen). Protein release was monitored with Size Exclusion Chromatography (SEC).

4.6 GBA endpoint activity assay

GBA containing samples were quantified for total protein using a BCA assay and diluted with VCB to fit into the assay range, between 5 ng/ml and 500 ng/ml of unmodified VPRIP. Protein activity was measured as percentage of GCase activity per mass unit of the unmodified VPRIV. This technique is used to assess the GCase.

In a 96-well plate (Nunc), 10 µl of each sample (in triplicates) were incubated for 1 hour with 90 µl of reaction buffer at 37°C. This reaction buffer was prepared containing 5mM 4-Methyllumbelliferyl β-D-glucopyranoside (a fluorescent GBA substrate, SIGMA), 22 mM Sodium Taurocholate (Sigma), 0.25% Triton X-100 (Fluka) in a 0.2M phosphate / 0.1M citrate buffer at pH 5.4. After 1 hour of incubation the reaction was stopped with 25 µl of stop solution consisting in a 0.25M glycine buffer at pH 10.4 (basified with NaOH 1M). Subsequently, aliquots of the resulting solution were measured by fluorescence spectroscopy on a Victor^3 1420-041 Multilabel Plate Reader (Wallac), $\lambda_{excitation}$: 365 nm; $\lambda_{emission}$: 450 nm. Fluorescence intensity arise from substrate degradation, and correlates with GCase activity.

5 Cell culture

5.1 Cell lines and maintenance

The culture conditions for all the cell lines used were 37ºC and 95% humidity. CO_2 concentration was set either at 5% or 10% as denoted in each cell line.

Adherent HeLa cells obtained from the American Type Culture Collection (ATCC® CCL-2™) were maintained in Minimum Essential Medium (MEM) α supplemented with GlutaMAX™ and 10% v/v of Fetal Bovine Serum (FBS, Invitrogen Inc) at 5% CO_2.

Adherent A431 cells obtained from the American Type Culture Collection (ATCC® CRL-1555™) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with GlutaMAX™ and 10% v/v of Fetal Bovine Serum (FBS, Invitrogen Inc) at 10% CO_2.

Adherent BE(2)-M17 (M17) obtained from the American Type Culture Collection (ATCC® CRL-2267™) were maintained in Opti-MEM® Reduced-Serum Medium supplemented with GlutaMAX™ and 10% v/v of Fetal Bovine Serum (FBS, Invitrogen Inc) at 5% CO_2.

BE(2)-M17 GBA-KO cells are a Knockout mutant of Adherent BE(2)-M17 for both alleles of GBA gene (Uniprot P04062). The cell line was characterized and validated by the Neurodegenerative Diseases Research Group at the Vall d'Hebron Research Institute (Barcelona). Cells were maintained in Opti-MEM® Reduced-Serum Medium supplemented with GlutaMAX™ and 10% v/v of Fetal Bovine Serum (FBS, Invitrogen Inc) at 5% CO_2.

5.2 EGF phosphorylation assays

Sub confluent A431 cells were plated into 6-well plates (Corning Inc) at a cell density around $4.5 \cdot 10^5$ cells per well (37.5% confluence). Cells were cultured in DMEM supplemented with GlutaMAX™ and 10% v/v of FBS until 75% confluence. Cells were then washed twice with DMEM without FBS (deprivation medium) and incubated for 24 hours in deprivation medium. Serum-deprived cells were then washed twice with deprivation buffer and stimulated with rhEGF-containing medium (concentration vary depending on experimental needs) for 15 minutes. Post treatment EGF removal as described in the next section. Cell extracts are subsequently used in Western Blot for anti-EGFR quantification.

5.3 Cell lysis

After treatments, plated cells were washed twice with cold PBS (4°C) and kept on ice. Remaining PBS was removed and 200 µl of M-PER® mammalian Protein Extraction Reagent with Halt™ Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific) were added in each well (volume for a 6-well plate) as a lysis buffer. Lysates were recovered by scrapping and frozen until their utilization (Thermo Scientific).

5.4 Scratch assays

The *in vitro* scratch assay is a simple and inexpensive method to study cell migration *in vitro*. By creating an artificial gap on a confluent cell monolayer, a "scratch", the cells of the edge will migrate to close the gap until cell-cell contacts are established anew (Liang, Park and Guan, 2007). This method can be used to mimic cell migration in wound healing *in vivo*.

Sub-confluent HeLa cells were plated into 6-well plates (Corning Inc) and cultured until confluence was reached. Confluent cells were washed twice with PBS and then serum deprived during 24h (MEMα-Ø). After deprivation, the cells were washed and a one-centimetre scratch was performed in each well with a 200 µl tip (Star Lab). Afterwards, cell debris is washed out with MEMα-Ø and the cells were treated with MEMα-Ø media with Growth factors at 50 ng/ml and nanoparticles (encapsulated protein was quantified and concentrations adjusted to 50 ng/ml of EGF). Plates are incubated for 8-24 hours at 37°C, but periodically taken out of the incubator for examination and capturing images.

5.5 Cell exposure to GBA-Conjugates and subcellular fractionation

Sub confluent M17 and M17 GBA-KO cells were (separately) seeded into 100 mm culture dishes (Corning). Cells were cultured in growth medium until 90% confluence was reached. Growth medium was removed from the dishes and replaced by nanoconjugates-containing medium that consist on Velaglucerase-PGA conjugates dispersed in Opti-MEM supplemented with GlutaMAX™ and 10% v/v FBS (growth medium). Conjugates final concentration varied among different experimental conditions. The cells were exposed to the nanoconjugates for 3 hours. Each condition was conduct in triplicates, in order to have enough cells for proper subcellular fractionation.

Subcellular fractionation was performed to analyse the GBA content and the GCase activity of different cellular fractions, mainly the lysosomes, as they are the target of the conjugates. During the fractionation procedure, cells were kept all the time at 4°C. Basically, the experimental procedure consist on washing the treated cells twice with cold PBS to ensure a complete non-internalized conjugates removal. Washed cells in PBS were scrapped and collected in Eppendorf tubes (cells from different dishes of the same condition were joined in this step). Collected cells were centrifuged at 800 g for 5 minutes and resuspended in 1 ml of 0.25 M Sucrose Buffer and 10 µl of Halt™ Protease Inhibitor Cocktail (Thermo Scientific). In order to break the plasma membrane without compromising the organelles integrity, cells were placed into a nitrogen cavitation chamber for cell disruption (Kimble Kontes). Pressure inside the chamber was slowly increased with nitrogen and kept at 2.4 bar for 7 minutes. Pressure was then abruptly relived to atmospheric pressure and the cellular content was collected into a Potter Elvehjem Homogenizer, where cells were grinded with a pestle until complete homogenization. The homogenate was centrifuged at 2500 g for 10 minutes, separating the organelle-containing cytosol from the post-nuclear pellet (PNP, which contain the nuclei and fragments of the plasma membrane). The cytosol fraction was centrifuged again at 17000 g for 15 minutes, so that mitochondria and lysosomes (pellet) were separated from the rest of the cytosol (supernatant).

5.6 Immunocytochemistry

As previously explained, immunochemistry techniques use the ability of antibodies to specifically recognise and attach to antigens to identify or quantify specific molecules. While western blot is an immunochemistry technique coupled to a protein separation procedure, immunocytochemistry is design to work on cell cultures. Coupled to cellular experiments or treatments, immunocytochemistry allows us to identify the presence of a specific protein (or any other molecule), quantify it and determine its cellular location. This technique is especially useful to see if there is co-localization of two molecules.

This technique is compatible with all the cell lines presented in this section. The cell line choice was made according experimental need in each case.

Sub confluent cells were seeded into sterile coverslip-containing 24-well plates (Corning) and cultured until desired density was reached. Cells were treated according to the experimental needs and processed for immunocytochemistry.

Treated cells were washed twice with DMEM without FBS at 37°C and fixed with 3% paraformaldehyde in DMEM for 5 minutes at 37°C. Fixed cells were then washed twice with PBS at 4°C, and kept cold until ready to permeabilize. Permeabilization was performed alongside blocking, by incubating the cells for 30 minutes at room temperature (RT) with blocking solution, a PBS buffer containing 0.2% non-fat dry milk (Central Lechera Asturiana), 0.1 M Glycine (Sigma), 1% BSA (Thermo Scientific) and 0.01% Triton X-100 (Fluka). Cells were washed 3 times with PBS.

Chapter III

Design of a therapy based in the encapsulation of EGF for treatment of Peptic Ulcers

CHAPTER III: DESIGN OF A THERAPY BASED IN THE ENCAPSULATION OF EGF FOR TREATMENT OF PEPTIC ULCERS

Protein encapsulation has been studied for many years as a strategy to overcome the shortcomings of protein therapeutics. Protein encapsulation not only can provide protection against environmental conditions that are harmful for proteins but also can improve their pharmacokinetic properties by a sustained release. Because of their properties, PLGA is a good candidate for protein encapsulation, as it is biocompatible, biodegradable, has great flexibility in processing, PLGA nanoparticles are able to perform a sustained release and it is already approved by the FDA and the EMA for parenteral administration

Herein, we study PLGA capability to encapsulate proteins with a therapeutic purpose. PLGA synthesis needs to be adapted so that protein structure and activity, and therefore its therapeutic capacity, are maintained. As a proof of concept, we have encapsulated a combination of two proteins with therapeutic interest in the healing process. The first one is the EGF a growth factor that stimulates de healing process and the other protein is protease inhibitor, the NvCI, that will protect EGF from proteolysis and improve its healing effect.

1 Coencapsulation of EGF and NvCI in PLGA nanoparticles

In order to test the protective effect that NvCI inhibition of metallocarboxipeptidases could have on EGF, coencapsulation of EGF and NvCI in PLGA nanoparticles was designed. PLGA nanoparticles, along the gastrointestinal tract are expected to protect its protein content against the harsh environmental conditions. Simultaneous release of NvCI and EGF is expected to inhibit metallocarboxypeptidase action against EGF and improve its performance in increasing the healing rate.

1.1 Synthesis parameters and characterization of PLGA nanoparticles

Poly(lactide-co-glycolide) (PLGA) nanoparticles of different physical characteristics (size, size distribution, morphology, zeta potential) can be synthesized by controlling the parameters

specific to the synthesis method employed. One of the most common methods, emulsion−solvent evaporation, is based on the shaping of the bulk material into nanostructures. Two types of the emulsion− solvent evaporation technique can be used: the single and double emulsion. The choice of each method depends upon the hydrophilicity of the drug to be encapsulated (Makadia and Siegel, 2011). In the single emulsion−solvent evaporation method, both drug (hydrophobic) and polymer are first dissolved in a water immiscible organic solvent, to form a single-phase solution. Then, this organic oil phase (O phase) is emulsified in water containing an appropriate emulsifier. If the molecule to encapsulate is hydrophilic, it is necessary to prepare a double emulsion. Here, the drug is dissolved in the aqueous phase (W phase). To induce size reduction to the nanoscale, sonication is used. The size reduction is induced through a high-energy process, breaking up the primary droplets of dispersed oil into nanosized droplets (Kentish et al., 2008; Mahdi Jafari, He and Bhandari, 2006). The subsequent removal of the organic solvent transforms droplets of dispersed phase into solid particles.

There are many factors that affect the outcome of PLGA nanoparticles synthesis. In order to achieve a particular set of reproducible characteristics, a thorough optimization of the synthesis parameters was performed. Inaccurate parameters may cause inhomogeneity in the resultant nanoparticles, as shown in Figure 3.1. There are some critical parameters such as sonication time or the concentration of the stabilizer in the O phase. The objective was to reduce polydispersion (Fig 3.1A) by adjusting sonication time and stabilizer concentration or avoid the presence of porous or holes in the resulting nanostructures (Fig. 3.1.B) by controlling the rate of solvent evaporation.

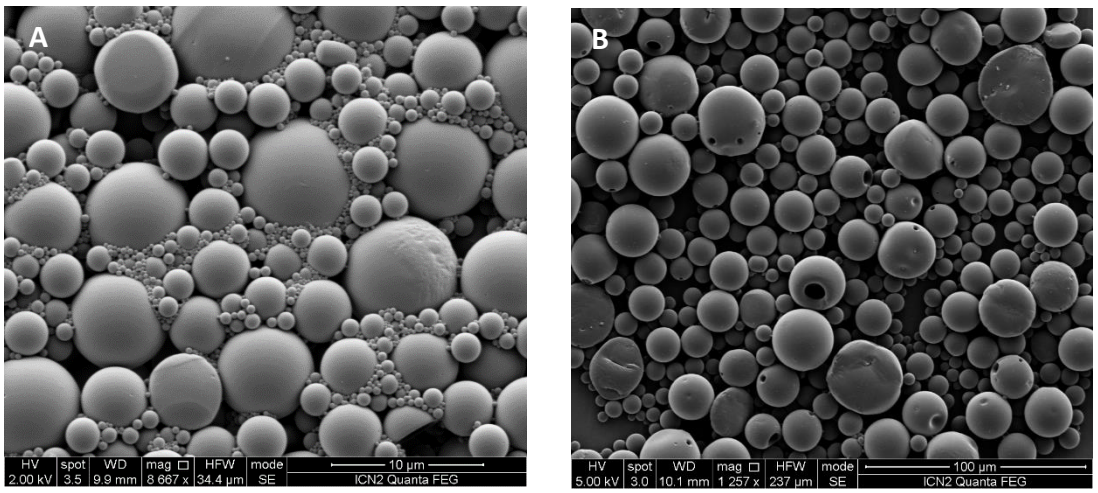

Figure 3.1. SEM microphotographs of PLGA nanoparticles. PLGA nanoparticles synthesised following unsuccessfully optimized parameters. (A) Nanoparticles were synthesised in short sonication times and

without an O phase stabilizer. (B) Nanoparticles were synthesised in short sonication times and solvent evaporation using rotary evaporator.

Optimization of synthesis parameters was intended to obtain a monodisperse population of nanoparticles, with a diameter around 200 nm, highly negative surface charge and with a high encapsulation efficiency. After testing many different synthesis parameters, the conditions were established as indicated in table 3.1.

Table 3.1. Parameters for PLGA nanoparticles.

Parameter	Value	Comments
Polymer	PLGA 50:50	719870, Sigma Aldrich
Concentration of PLGA in O	33,3 mg/ml	
Organic Solvent	Dichloromethane	
W_1 buffer	PBS	
Stabilizer for O phase	Span 80, 4% v/v	
Stabilizer for W_2 phase	PVA 2% (low mw), 1 M NaCl	
Ratio W_1/O	1:10 (volume)	
Ratio O/W_2	1:12.5 (volume)	
Sonication amplitude	25% (in microtip)	
W_1/O sonication time	90 s	
O/W_2 sonication time	90 s	
Solvent evaporation	3 hours	Under magnetic stirring
Centrifugation speed	30.000 g	
Centrifugation time	45 minutes	3 washes

1.2 Encapsulation of EGF and NvCI.

In order to determine the properties of a nanoparticle a thorough characterization must be performed. Parameters measured to determine the chemical and physical properties of the nanoparticles include particle shape and size, porosity, particle size distribution, zeta potential, hydrodynamic diameter, aggregation, encapsulation efficiency and release. In order to determine those parameters many techniques are carried out. As commented, double-emulsion solvent evaporation technique is a suitable choice for encapsulating hydrophilic drugs (including proteins). Using this method, EGF and NvCI were encapsulated in PLGA separately and in combination. In the case of EGF@PLGA nanoparticles, 75 μL of an aqueous solution of 3 mg/mL EGF in PBS (W_1 phase) was added dropwise to 750 μL of organic phase containing PLGA polymer dissolved in dichrolomethane (33.3 mg/mL). Subsequent sonication for 90 seconds yielded the first emulsion, a water-in-oil emulsion. Then, 9.4 mL of the 2% (w/v) PVA aqueous solution with 1M NaCl (W_2 phase) was added dropwise to the prepared emulsion and sonicated again for 90 seconds. As a result, a water-in-oil-in-water emulsion is formed. The mixture was stirred (500 rpm) at room temperature for 4 hours in order to completely evaporate the organic solvent. It is important to note that this transfer step must be completed very quickly, in order to avoid PLGA NPs aggregation before being stabilized by the surfactant (PVA). The resulting suspension was centrifuged and washed 3 times at 30000 g at 4 °C. For NvCI@PLGA the same procedure was performed, being the W_1 phase 75 μl of 3 mg/ml NvCI in PBS. Finally, for EGF/NvCI@PLGA the synthesis was performed following the same procedure with a 75 μl of 3 mg/ml EGF, 3 mg/ml NvCI in PBS as a W_1.

2 Nanoparticle characterization

2.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is a type of electron microscopy that provides information about the surface topography of a sample. With that information, the shape and porosity of a nanoparticle can be determined. Although the diameter of the nanoparticles can also be measured from SEM microphotographs, sample processing requires the samples to be dried up, and thus their diameter will be altered. Characterization of the size of nanoparticles must be complemented with the measuring of the hydrodynamic diameter with DLS.

Characterization of nanoparticles synthesised following the parameters of Table 3.1 showed reproducible nanoparticles, mostly spherical and non-porous. No information of aggregation can be extracted from SEM microphotographs as desiccation of the nanoparticles produces agglomeration, which can be observed in fig. 3.2.

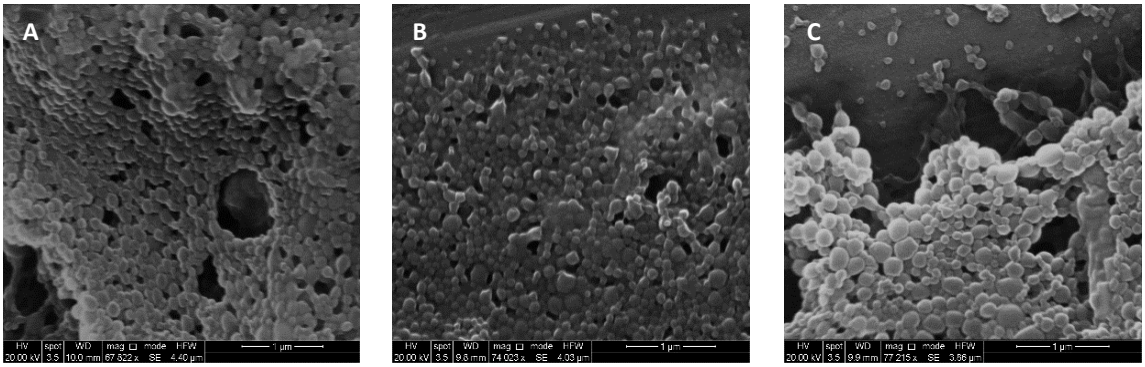

Figure 3.2. SEM microphotographs of PLGA nanoparticles. PLGA nanoparticles synthesised following the parameters from Table 3.1. (A) W_1 contains PBS 1X. (B) W_1 contains 3 mg/ml of EGF in PBS. (C) W_1 contains 3 mg/ml of NvCI in PBS.

The size of the nanoparticles calculated from the SEM microphotographs is highly reproducible and is not significantly affected by protein concentration in the W_1 phase.

Table 3.2. PLGA nanoparticles diameter regarding protein content in W_1 phase.

#	W_1 phase	Diameter (nm)
PBS@PLGA	**PBS 1X**	152.7 ± 7.0
EGF@PLGA	**EGF 3 mg/ml in PBS**	157.0 ± 6.6
NvCI@PLGA	**NvCI 3 mg/ml in PBS**	148.6 ± 6.6
EGF/NvCI@PLGA	**EGF 3 mg/ml + NvCI 3 mg/ml in PBS**	153.7 ± 14.1

2.2 Dynamic Light Scattering (DLS)

DLS is used to determine the size distribution profile of small particles in suspension. The information obtained by DLS measurement allows us to calculate the hydrodynamic diameter

(Z-Average) and the Polydispersity Index Value (PdI), a dimensionless measure of the size distribution of the nanoparticles. In the used equipment (Zetasizer Nano ZS, Malvern), PdI ranges from 0.1, being samples with PdI values under 0.08 considered "monodisperse", although values under 0.3 indicate an acceptable polydispersity for pharmaceutical uses of the nanoparticles.

Electrophoretic Light Scattering (ELS) is a technique based on DLS. It is used to measure the Zeta Potential (Z-potential), or the magnitude of the electrostatic between particles by which we can estimate the surface charge of the nanoparticles.

Table 3.3. PLGA nanoparticles hydrodynamic diameter, PdI and Zeta Potential regarding protein content in W_1 phase. Nanoparticles were diluted at 5 μg/ml in MilliQ water.

#	W_1 phase	Z-Average (nm)	PdI	Z-Potential (mV)
PBS@PLGA	**PBS 1X**	205.9 ± 1.1	0.186	-26.2 ± 1.3
EGF@PLGA	**EGF 3 mg/ml in PBS**	266.4 ± 5.3	0.203	-20.4 ± 0.5
NvCI@PLGA	**NvCI 3 mg/ml in PBS**	244.8 ± 8.1	0.157	-24.3 ± 0.5
EGF/NvCI@PLGA	**EGF 3 mg/ml + NvCI 3 mg/ml in PBS**	208.2 ± 8.1	0.123	-25.1 ± 0.7

Nanoparticles with Z-potential between -20 and +20 mV are described to be unstable and tend to aggregate. Higher internalization rates for negatively charged nanoparticles has been described (Lunov et al., 2011). Highly negative values of surface charge are intended for a better stability and internalization of the nanoparticles.

Although Zeta potential is better measured in MilliQ water (the presence of solutes interferes with the ELS measurement, data not shown), the hydrodynamic diameter and the stability of the nanoparticles are more accurately measured in a pseudo-biological media such as PBS at pH 7.4 or PBS supplemented with BSA. Proteins are described to bind immediately onto the surface of nanoparticles upon introduction into a physiological environment (Aggarwal et al., 2009). PBS supplemented with BSA, as it mimics a physiological environment with the presence of plasma proteins, is useful for studying opsonisation of nanoparticles in biological media.

Table 3.4. PLGA nanoparticles hydrodynamic diameter regarding protein content in W_1 phase and resuspension media. Nanoparticles were diluted at 5 μg/ml in MilliQ water (H_2O), in PBS 1X and PBS 1X with 0.5 mM of BSA.

#	W_1 phase	Z-Average (nm)		
		In H_2O	In PBS	In PBS + BSA
PBS@PLGA	**PBS 1X**	205.9 ± 1.1	258.6 ± 2.2	222.0 ± 11.9
EGF@PLGA	**EGF 3 mg/ml in PBS**	266.4 ± 5.3	232.2 ± 6.4	302.8 ± 5.9
NvCI@PLGA	**NvCI 3 mg/ml in PBS**	244.8 ± 8.1	255.7 ± 9.8	300.6 ± 14.6
EGF/NvCI@PLGA	**EGF 3 mg/ml + NvCI 3 mg/ml in PBS**	208.2 ± 8.1	213.0 ± 6.3	265.0 ± 8.2

Although measurement of the hydrodynamic diameter was less reproducible than direct diameter measurement by SEM, it is clear that nanoparticles in PBS supplemented with BSA experiment a hydrodynamic radius increasing that can be associated to the opsonisation of the nanoparticles by BSA and formation of the protein corona.

2.3 Encapsulation Efficiency determination. Protein detection and quantification.

When encapsulating proteins, being able to detect and quantify the encapsulated protein is crucial for the determination of the Encapsulation Efficiency (EE) and drug release. However, quantifying the encapsulated protein can be challenging for many reasons. Most of the techniques developed for protein quantification, and used for many decades, have been designed and optimized to work in biological media, either *in vitro* or *in vivo*. Unfortunately, the physicochemical nature of nanoparticles and their components can interfere or distort those techniques. In this scenario, alternative analytical techniques that facilitate the quantification of the encapsulated protein are necessary.

As it has been introduced in Chapter 2, the encapsulation efficiency determines the percentage of encapsulated protein over the total amount of protein in the internal phase (W_1 phase). There are two main methodologies to calculate the encapsulation efficiency (EE),

either directly or indirectly. A direct determination of EE measures the amount of encapsulated protein while an indirect determination measures the non-encapsulated protein in the supernatant and calculates the encapsulated protein by subtraction from the total amount. All the methods used in this project were indirect determinations, as the main setback for direct determination is that the protein is still encapsulated inside the nanoparticles, which hinders the interaction with the detection and quantification methods. PLGA nanoparticles can be broken by incubation in DMSO or NaOH, but because of the inconveniences showed for any of the detection methods attempted, the indirect method was deemed more reliable in any case.

UV spectroscopy is routinely used for approximate protein quantification. However, when a pure protein sample is measured and there are no UV-absorbing contaminants, if the molar extinction coefficient of the protein is known, the Lambert-Beer law can be used to accurately quantify the amount of protein. UV absorbance at 280 nm and at 214 nm, caused by the aromatic amino acids and the peptide bonds respectively, are characteristic for protein quantification. Although successfully used for quantification of the pure protein solution stocks utilised for the synthesis of nanoparticles, this method alone resulted ineffective for protein quantification after encapsulation. The presence of contaminants in the samples or the encapsulation of more than one protein forbid precise and specific protein quantification, although UV absorbance is used as a detection method coupled in protein separation techniques.

BCA and Bradford assay, as described in the Experimental Procedures section, are colorimetric methods for quantification of total protein. The use of Bradford assay was quickly discarded when quantifying the small proteins EGF and NvCI, and because of the interference of PLGA and PVA in the technique, it was also discarded for any protein measure in PLGA nanoparticles. The BCA assay remained unaltered by PLGA and PVA contaminants, but as a total protein quantification method, it is unable to individually quantify two or more proteins in a solution. BCA was successfully used as a complementary quantification method in the preparation of pure protein stocks for encapsulation.

In order to independently quantify two proteins in the same sample (as needed in the determination of the EE in nanoparticles encapsulating both EGF and NvCI), either a separation method or a specific detection method had to be used, so two distinguishable signals, one for each protein, could be measured.

SDS-PAGE is a common method used for protein separation by molecular weight. In order to independently quantify EGF and NvCI from a double encapsulation sample, two main difficulties arose. First of all, the samples used for EE determination, as they were taken from the supernatants after centrifugation of freshly synthesised nanoparticles, had 1M of NaCl and 2% of PVA, which greatly disturbed the mobility of the proteins through the gel, showing vague, broad bands. Secondly, EGF and NvCI have a very similar molecular weight, around 6 kDa, and because of the disturbance in the protein mobility the bands of both proteins overlap. Staining the gel with unspecific protein stains like coomassie, blue safe or silver staining is not able to distinguish both proteins from one another. Performing a western blot with anti-NvCI and anti-EGF antibodies, although being able to detect non-encapsulated NvCI and EGF standards, was also found ineffective for protein detection in the supernatants, as the protein levels were under the detection limit.

RF-HPLC was successfully used to detect and quantify EGF and NvCI (Figure 3.3). A characteristic single peak for NvCI was detected after 28.89 minutes and its area after integration directly correlated with NvCI amount. EGF showed an elution profile with several peaks, four of them clearly delimited and directly proportional with EGF amount. The peak at minute 32.35 showed the most activity and was selected for protein quantification.

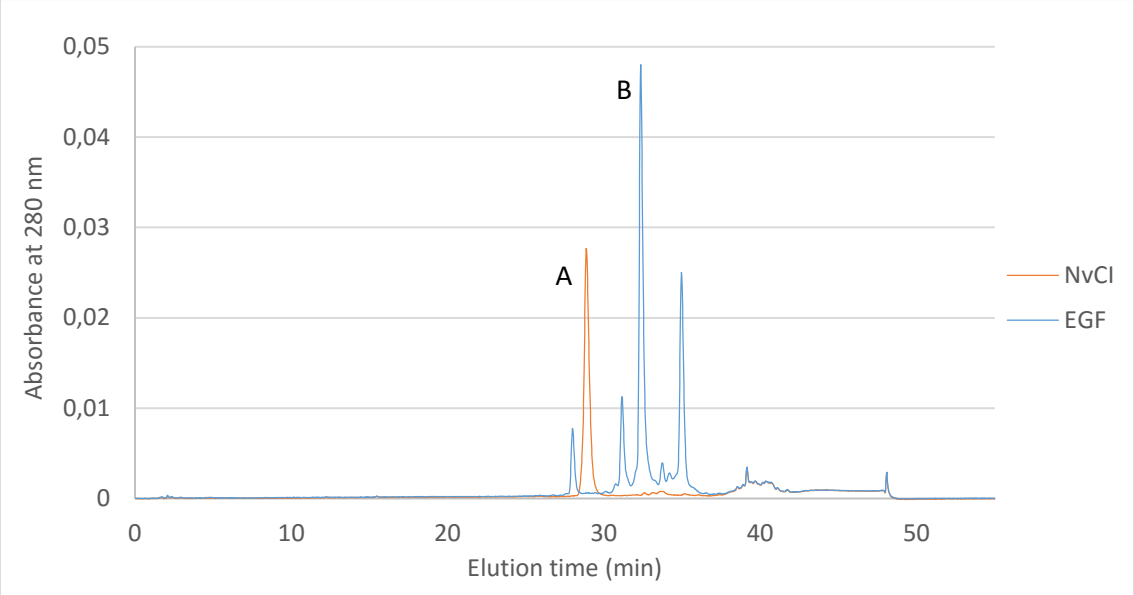

Figure 3.3. Quantification of NvCI and EGF by RP-HPLC chromatography. EGF and NvCI stocks were acidified with 0.1% v/v TFA and loaded into a Nova-Pak C8 cartridge. Absorbance at 280 nm for protein detection was monitored throughout time (A) NvCI elution peak at minute 28.89. (B) EGF elution peak at minute 32.35.

Quantification of EGF and NvCI with RF-HPLC was used to determine the Encapsulation Efficiency (EE). Although EE determination showed slight variability amongst batches of nanoparticles, no significant differences of EE are observed between encapsulation of EGF and NvCI, either when encapsulated alone or together, as shown in Table 3.5. For the initial concentrations of protein in W_1, either EGF or NvCI EE was around 40%, so when co-encapsulated together, and considering their similar molecular weight, EGF and NvCI were essentially in equimolar concentration.

Table 3.5. Encapsulation Efficiency of different batches of nanoparticles regarding protein content in W_1 phase.

#	W_1 phase	EE NvCI	EE EGF
PBS@PLGA	**PBS 1X**	-	-
EGF@PLGA	**EGF 3 mg/ml in PBS**	-	36.5% ± 4.0%

NvCI@PLGA	**NvCI 3 mg/ml in PBS**	42.7% ± 3.8%	-
EGF/NvCI@PLGA	**EGF 3 mg/ml + NvCI 3 mg/ml in PBS**	37.5% ± 2.77%	40.33% ± 1.7%

2.4 Protective effect of NvCI against metallocarboxypeptidase degradation of EGF

Previous studies performed by our research group studied the protective effect of NvCI on EGF against degradation by digestive metallocarboxypeptidases (MCPs) (Alvarez Fernández, 2015). In a plain *in vitro* assay, recombinant human Epidermal Growth Factor (rhEGF) was incubated with a mixture of the four digestive MCPs (30 nM of CPA1 and CPA2, and 50 nM of CPB and CPO) , with and without NvCI. As observed in Figure F304, it takes less than 15 minutes for the MPCs mixture to cleave the C-terminal regions of the predominant EGF_{1-51} and EGF_{1-52} forms into the less active EGF_{1-47} and EGF_{1-48} forms. The presence of NvCI successfully inhibits MPCs degradation and protects EGF_{1-51} and EGF_{1-52} from cleavage. For two hours, in presence of NvCI, most of the active EGF_{1-51} and EGF_{1-52} forms are maintained undigested.

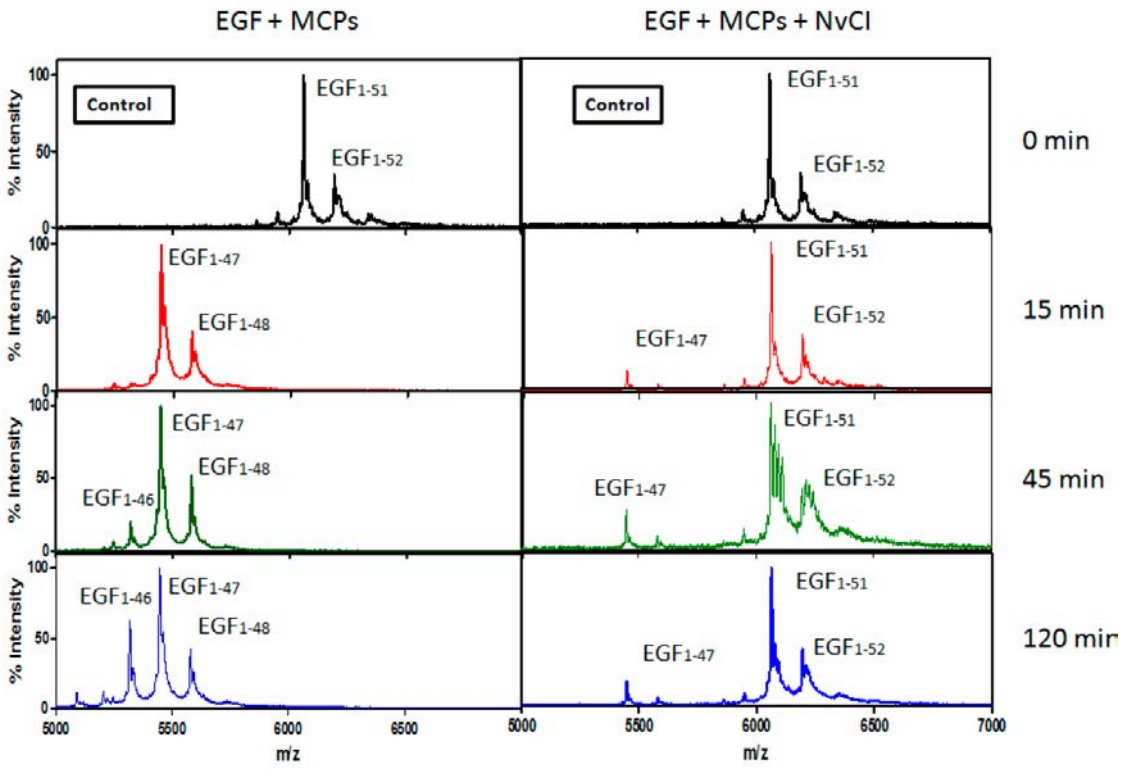

Figure 3.5. Proteomic analysis of rhEGF after incubation with MPCs at 37°C at pH 7.5 in presence (right) or absence (left) of NvCI at 100 nM. Representative MALDI-TOF spectra of rhEGF (EGF1-52 and EGF1-51)

after incubation with a mixture of 30nM hCPA1, 30 nM hCPA2, 50 nM pCPB and hCPO. Representative spectra is shown from each time point. Image from Roberto Fernández (Alvarez Fernández, 2015).

2.5 Effect of digestion and encapsulation on EGF biological activity

Biological activity of EGF was tested by phosphorylation of EGF receptors (EGFR) from A431 cells. Levels of phosphorylated EGFR (pEGFR) after EGF exposure allows us to assess the levels of protein activity retained by the EGF.

Untreated EGF at different conditions (20 nM and 100 nM) and EGF digested with MCPs for 120 minutes (at the same conditions than in Figure 3.5) were used to stimulate A431 cells. Protein extracts after stimulation were analysed by SDS-PAGE and western blot using an anti-pEGFR antibody. Phosphorylation levels were quantified by densitometry, showing that untreated EGF have dose-dependent biological activity, but this activity is severely reduced after digestion by MCPs, as indicated in Figure 3.6.A.

Encapsulation of EGF in PLGA nanoparticles doesn't seem to impair its biological activity, as it can be observed in Figure 3.6.B. NvCI also maintains its biological activity after encapsulation and subsequent release (data not shown) (Alvarez Fernández, 2015).

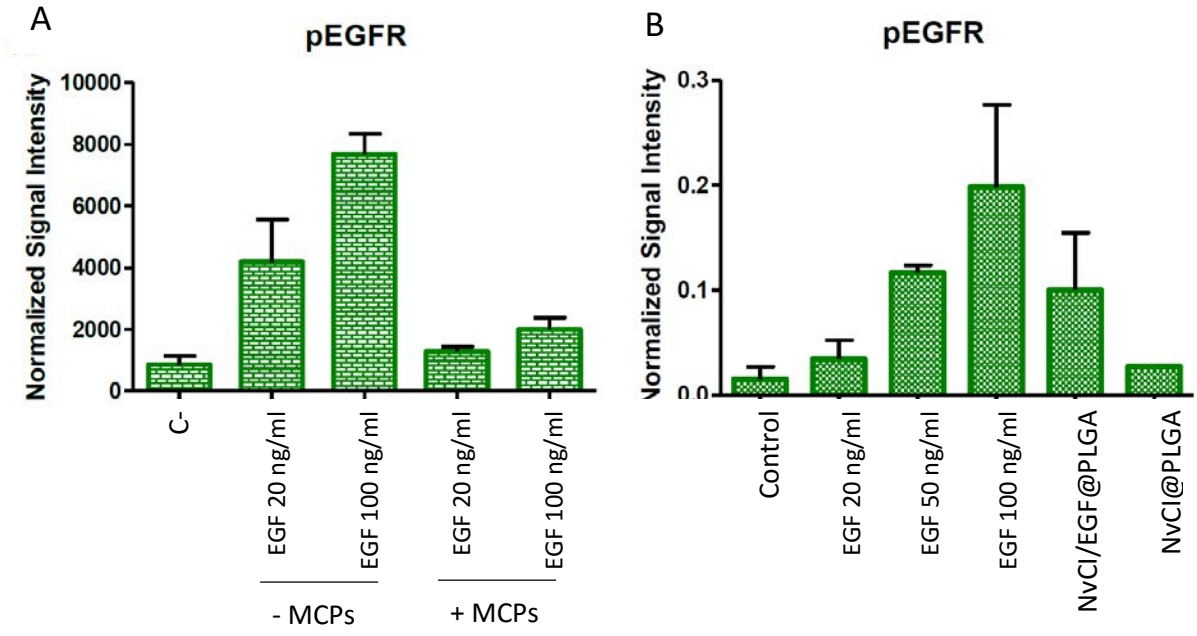

Figure 3.6. Densitometric analysis of pEGFR levels in A431 cells after treatments with EGF. pEGFR levels were normalized with α-tubulin levels, that acted as a loading control. (A) pEGFR levels after stimulation with non-encapsulated EGF, untreated and digested with MCPs for 120 minutes (B) pEGFR levels after stimulation with free EGF at different concentrations (EGF 20 ng/ml, 50 ng/ml and 100 ng/ml), and EGF released from nanocapsules (NvCI/EGF-NC, equivalent to EGF 20 ng/ml). Untreated cells and cells treated with NvCI extracted from nanocapsules were used as controls. Figure from Roberto Fernández (Alvarez Fernández, 2015).

2.6 Protective effect of PLGA nanoparticles during the gastrointestinal digestion

In order to assess the protective effect that encapsulation in PLGA and coencapsulation with NvCI would have on EGF, EGF ability to maintain its biological activity had to be tested. In this particular case, we want to know if PLGA nanoparticles, administrated orally, would be able to deliver its cargo in the gastrointestinal lumen and if that cargo, once released, would maintain its biological activity. Unlike in a release for parenteral administration, where studying the release throughout time provides useful information, nanoparticles administrated orally intended to deliver their cargo in the stomach or the proximal duodenum, only will remain in those areas for few hours and will proceed with their path throughout the gastrointestinal tract.

A gastrointestinal simulation was designed, where acidic pH and the presence of pepsin mimic the stomach environment for 2 hours, and a slightly basic pH and the presence of pancreatin mimic the duodenum environment for 3 hours. Nanoparticles that underwent a gastrointestinal simulation were subsequently exposed to serum-deprived A431 cells to evaluate its capability to phosphorylate EGFR, and to serum-deprived, confluent HeLa cells, with a scratch inflicted on the culture, to assess the effect of the nanoparticles in the healing rate in a scratch assay.

Nanoparticles containing EGF and NvCI (EGF/NvCI@PLGA), nanoparticles containing only EGF (EGF@PLGA) or only NvCI (NvCI@PLGA) and a control nanoparticle without encapsulated protein (PBS@PLGA) underwent gastric simulation as described in the experimental procedures section, and samples of the same nanoparticles were kept undigested as controls.

As it can be observed in Figure 3.7, no significant stimulation of the A431 cells was detected in any of the assayed conditions (except for the positive controls). No biologically active EGF

seems to be released by the end of the gastrointestinal simulation, and thus no EGFR phosphorylation is observed.

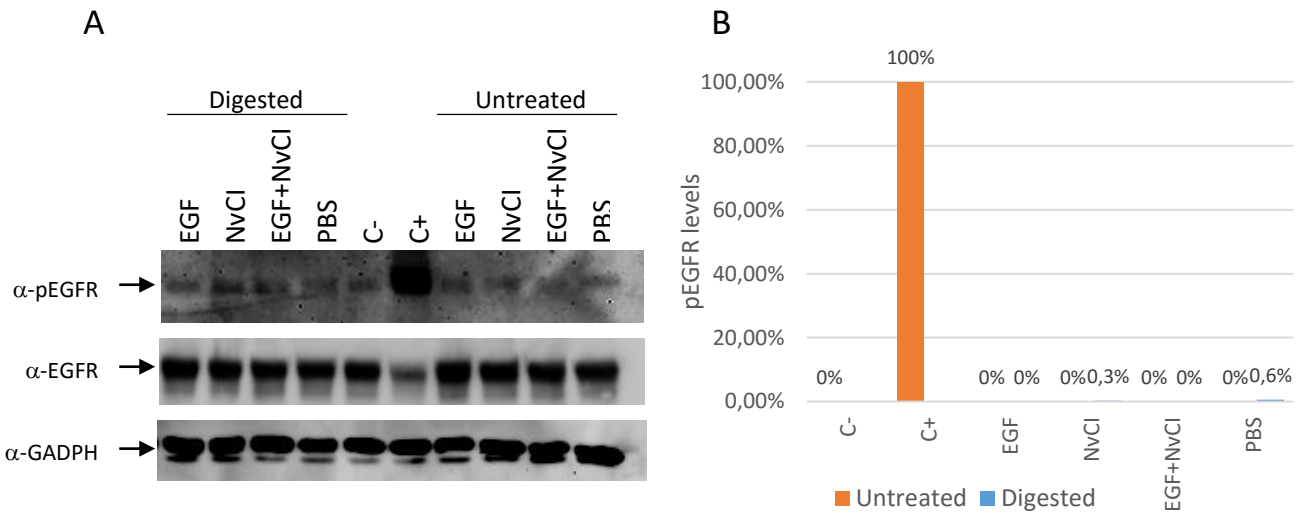

Figure 3.7. PLGA nanoparticles underwent a gastrointestinal simulation. Samples after digestion (and their untreated counterparts) were used to stimulate for 15 minutes A431 cells, which had been deprived from serum for the previous 24 hours. EGFR phosphorylation levels were analysed by western blot (A) and the ratio of EGFR phosphorylation (B) was calculated as a percentage of the positive control (C+, A431 cells were stimulated with non-encapsulated, undigested EGF at 50 ng/ml) and a negative control (C-, untreated A431 cells) was used as a 0%.

2.7 Epithelial wound healing in scratch assays

Scratch assay was tested as an inexpensive model for wound healing in epithelial tissues. As described in the Experimental Procedures section, cultures of confluent HeLa cells were used as an epithelial tissue model, a scratch made with a pipette tip could be used to simulate a wound. Testing the scratch assays stimulating serum deprived cells with EGF showed that cell migration was responsive to stimulation within 15 hours. Figure 3.8 shows cell migration after stimulation with 50 ng/ml of EGF, using deprived, unstimulated cells as a negative control.

Much longer exposures could not be tested as besides the scratched area, the rest of the culture was confluent and started to decay. In figure 3.8 can be observed that after 48 hours of stimulation cells began to detach.

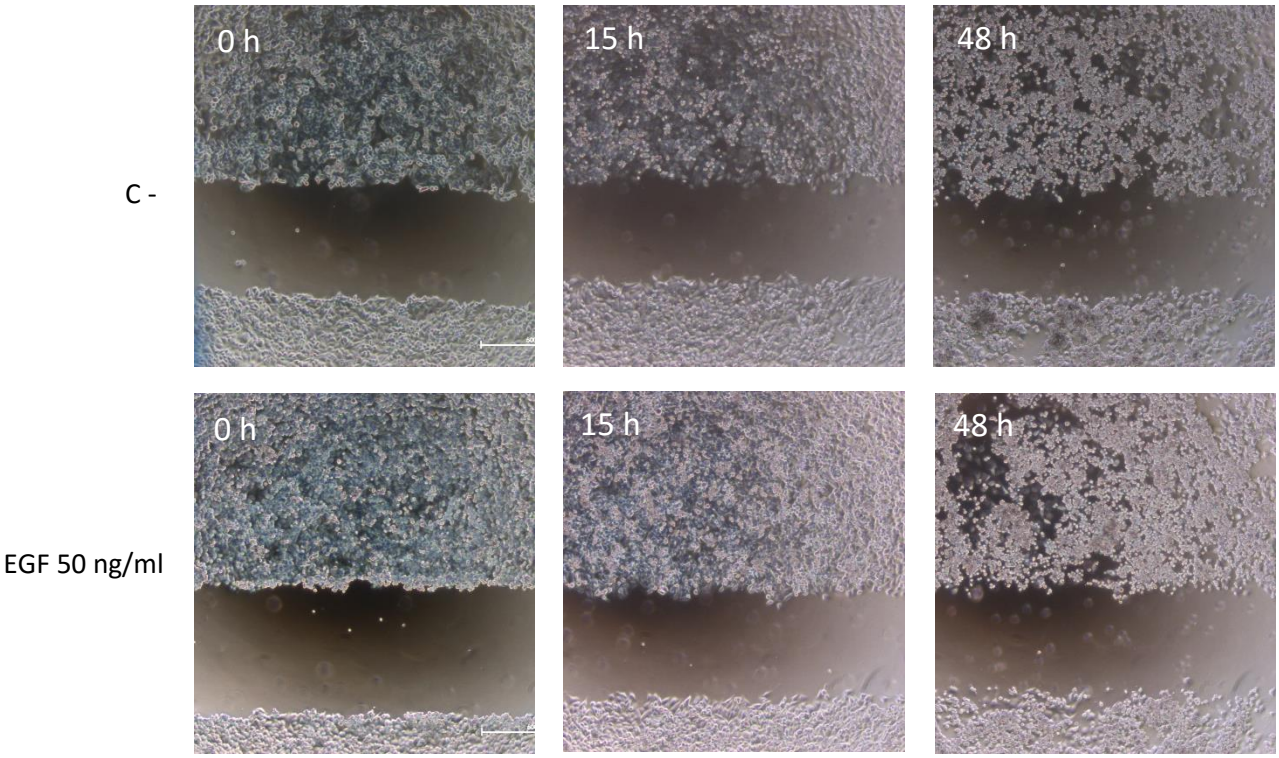

Figure 3.8. Confluent HeLa Cells were deprived from serum for 24 hours and an scratch simulating an epithelial wound was performed. Cells were stimulated with 50 ng/ml of EGF (a negative control remained untreated) and cell migration was monitored by optical microscopy for 48 hours.

The effects of nanoparticles in the healing rate of a confluent HeLa cell culture were not more promising than the results on EGFR stimulation. The nanoparticles that underwent gastrointestinal simulation and their untreated counterparts were exposed to confluent HeLa cell cultures that had been deprived from serum for 24 hours. Prior to exposure, a scratch was made in the cell cultures to mimic an open wound an epithelial tissue, so that the healing rate can be assessed by cell migration. In Figure 3.8 we can see the cell migration of HeLa cells after exposure to the nanoparticles, either untreated (Figure 3.9.A) or digested (Figure 3.9.B). Again, although HeLa cells are responsive to EGF stimulation, as can be seen after stimulation with non-encapsulated, untreated EGF at 50 ng/ml (C+), stimulation with nanoparticles with encapsulated EGF fail to increase the healing rate of the cells.

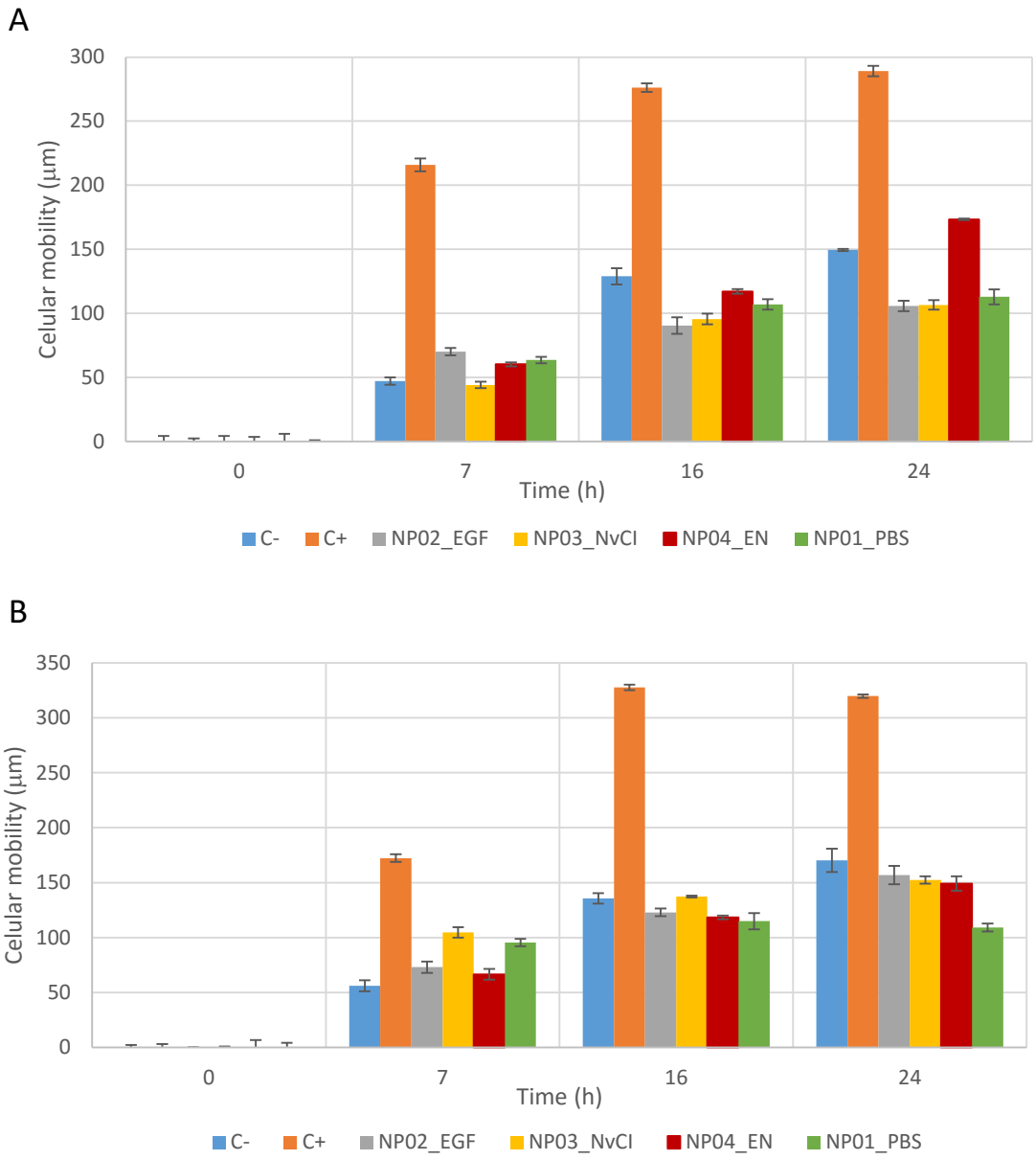

Figure 3.9. Cell migration of HeLa Cells during a scratch assay. Confluent HeLa cells underwent serum starvation for 24 hours and then a scratch was made on the surface of the culture. Cultures were stimulated with nanoparticles containing EGF, NvCl, both EGF and NvCl and a control without encapsulated protein. A negative control (C-) kept cells untreated and a positive control was perform with stimulation with non-encapsulated, undigested EGF at 50 ng/ml. Concentration of encapsulated protein was adjusted to 50 ng/ml. (A) HeLa cells were stimulated with undigested nanoparticles for 24 hours. (B) HeLa cells were stimulated with digested nanoparticles for 24 hours. Cell migration was analysed through optic microscopy, using the software NIS-Elements Viewer.

Chapter IV

IMPROVEMENT OF THE ENZYME REPLACEMENT THERAPY FOR GAUCHER DISEASE TREATMENT

CHAPTER IV: IMPROVEMENT OF THE ENZYME REPLACEMENT THERAPY FOR GAUCHER DISEASE TREATMENT

1 Encapsulation of GBA with PLGA, the case of a failed proposal

The main objective of this thesis has been to develop and optimize the protein nanoencapsulation process to obtain a nanodrug composed by enzymatically active Velaglucerase alfa. All the efforts have been focused on the protection of the enzyme and its transport to the lysosome for restoring the lysosomal and autophagic functions in dopaminergic neurons of Gaucher's disease patients through enzyme replacement therapy (ERT). Thus, various attempts were made to develop the encapsulation of Velaglucerase alfa in PLGA nanocapsules and analyse the stability of the enzymatic activity during and after encapsulation process and study of lysosomal targeting and drug release profiles *in vitro.*

As explained previously, two of the main inconveniences concerning Velaglucerase alfa ERT is the inability of the protein to cross the Blood-Brain Barrier (BBB) (Horowitz et al., 2016) and its rapid clearance from systemic circulation (Morris, 2012). PLGA encapsulation was explored as a robust solution in order to improve Velaglucerase stability while in systemic circulation and as a carrier to deliver compounds across the BBB (Cai et al., 2016).The first attempt by our group to overcome the shortcomings of Enzyme Replacement Therapy (ERT) using Velaglucerase alfa starts in 2014 (Martínez, 2016).A protocol for the synthesis of PLGA nanoparticles using the double emulsion and evaporation method was set up and optimized for protein encapsulation (Martinez, 2016). In a typical experiment, 25 mg of 50:50 PLGA were dissolved in 750 µL of dichloromethane (DCM) containing 3% of Span-80. Subsequently, 75 µL of an aqueous solution containing the protein (2.5 mg / mL) was added and the emulsion was sonicated for one minute in an ice bath. The resulting emulsion was added to an aqueous solution containing 2% PVA and sonicated again for 1 minute. Finally, then the solution was stirring for 4 hours to evaporate the DCM, centrifuged and lyophilized. This process was used for the encapsulation of Velaglucerase and lysozyme. The lysozyme was used as control since is a cheap protein of low molecular weight (14.4 kDa) and contains many disulphide bonds in the structure, which confer it a high stability.

The results indicated that the encapsulation efficiency of the proteins in the PLGA nanocapsules was satisfactory (≥ 35%), and the release from the capsules showed a good

control release pattern for several days (Figure 4.1). Moreover, the in vitro studies corroborated that this nanosystem was able to deliver its cargo into the lysosomes in mouse dopaminergic neurons. However, the results indicated large differences in enzyme stability. While the lysozyme maintained almost all its enzymatic activity, in the case of GBA high rates of Velaglucerase aggregation were observed and no GBA activity was detected neither in the release assays nor in the *in vitro* assays .

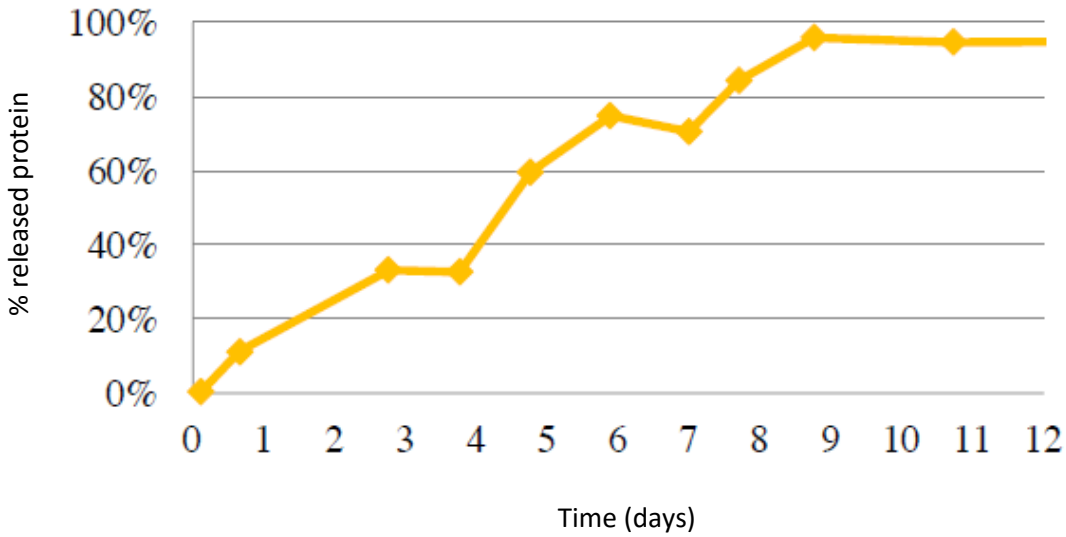

Figure 4.1. Lysozyme@PLGA nanoparticles at 1 mg/ml in VCB buffer pH 4.5 underwent a release assay in a dialysis bag. Protein was quantified by BCA assay (Martínez, 2016).

Detailed studies of the factors that could affect the stability during the encapsulation process corroborate the high sensitivity of Velaglucerase to environmental factors (temperature, pH and ionic strength) combined with the harsh conditions of PLGA nanoparticles synthesis procedure, such as high energy sonication and exposure to organic solvents, lead to a complete loss of enzymatic activity. Many attempts were made to overcome this dramatic lack of enzymatic activity during the encapsulation process (i.e. change of solvents, use of mild reaction conditions, use of protein stabilizers), but were found unsuccessful.

1.1 Fighting Velaglucerase instability. Need for a milder approach

After unsuccessfully trying to protect Velaglucerase from irreversible activity loss during encapsulation in PLGA nanoparticles, this system was discarded for encapsulation of large, sensitive proteins. In this scenario, a new system had to be found in order to fulfil the needs that PLGA encapsulation had failed to accomplish. In basis of the previous results and

knowledge, we try to find a process suitable to protect the Velaglucerase from aggregation and degradation processes during modification, and that allows reach the lysosomes where the enzyme can be released in a control manner maintaining its initial enzymatic activity. Thus, we needed a modification or encapsulation system that met the following requirements:

- The process of encapsulation/coating should not comprise the stability and enzymatic activity of the enzyme.
- The encapsulation or protective coating should generate a nanoconstruct able to reach the lysosomes.
- Once the Velaglucerase is into the lysosomes, the enzyme should be released in a control manner and recover the enzymatic activity.
- The final system should be further modified for specific delivery or BBB crossing.

For this purpose, we considered as an interesting approach for Velaglucerase-based ERT to conjugate the protein with a Poly(L-Glutacic acid) (PGA) polymer using a Polymer Masked-Unmasked Protein Therapy (PUMPT) technique (Talelli and Vicent, 2014).

The main advantages of this method is that uses mild conditions during protein modification (there are no high energy agitations nor exposure to high rates of organic solvents, and pH conditions can be adapted to some extent to protect the enzyme), the coating can stabilize the enzyme during systemic circulation and the chemical bond between the protein and the polymer (disulphide bond) is sensitive to reduction in intracellular environment (Talelli and Vicent, 2014), which can permit the protein release once the lysosome is reached. Besides, as PGA is basically a polypeptide chain, the N-terminal, C-terminal positions or side chains can be modified to be functionalized with other functional molecules.

Previous studies (Talelli and Vicent, 2014) showed as the concept of PUMPT has been successfully implemented in lysozyme modification. The results indicated that the protein activity had been successfully masked after coating, and most of the protein activity could be recovered after enzyme release in reductive conditions. Taking into account this seminal work, we decided to implement this method for the stabilization and protection of Velaglucerase and optimized it in order to perform the adaption of the process for this highly sensitive protein.

2. PUMPT method for Velaglucerase conjugation

The starting point for Velaglucerase modification with the PUMPT technique was the aforementioned work published by Talelli and Vicent in 2014. In this article, a PGA polymer (a 200-residue L-glutamic acid polypeptide) was modified by adding pyridyl dithiol groups (PD) in around 7% of the glutamic acid (Glu) residues present on the side chain. In this case, Lysozyme was modified with SATP in order to introduce acetate-protected thiol groups in a small number on lysine residues. After SATP modification, thiol groups were deprotected with a deacetylation solution (0.5 M hydroxylamine, 25 mM EDTA in PBS pH 7.4) and exposed to PGA modified with PD (PGA-PD), and disulphide bonds were formed between the thiols and the PD.

This procedure, optimized for lysozyme, was modified adequately for adapting it to the coating of Velaglucerase. The main differences that justify the modification of the initial protocol were:

- There are some significant differences regarding protein stability that may cause activity loss in Velaglucerase during the coating procedure, as shown in previous studies concerning encapsulation of the enzyme with PLGA. Temperature, pH conditions, buffer composition and reaction times need to be optimized for Velaglucerase.
- In order to find an optimal degree of lysozyme modification, a SATP/lysozyme ratio was studied based on the number of lysines in the lysozyme sequence (six lysines). Velaglucerase is a much larger protein and contains 22 lysines (20 of which are on the protein surface), as seen in Figure 4.2. Taking into account this significant difference, an optimization of the functionalization and coating process was performed.

A thorough optimization of the conjugation procedure was carried out minimizing the affectation of the structure and properties of the enzyme. In the following sections, the detailed study of the different parameters was described.

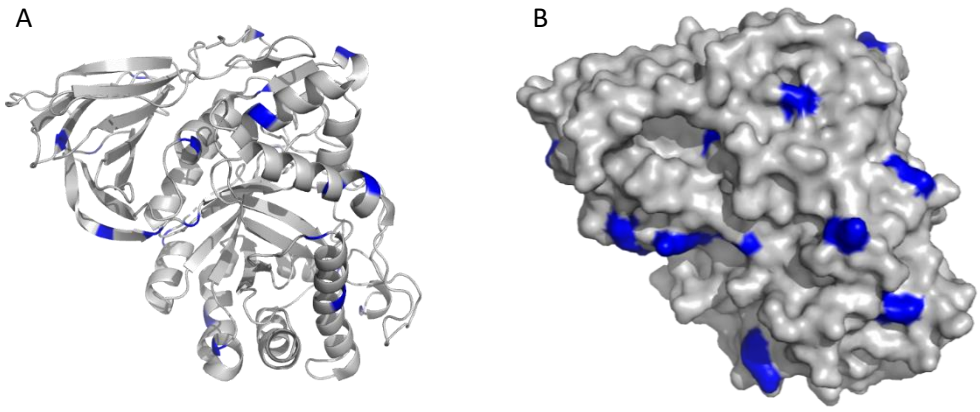

Figure 4.2. Representative structure of Velaglucerase alfa. PDB entry 2WKL, Velaglucerase alfa, was analysed and processed with Pymol 2.1.1. (A) Ribbon representation of Velaglucerase alfa. Lysine residues are highlighted in blue (B) Surface representation of Velaglucerase alfa. Lysine residues are highlighted in blue.

2.1 Velaglucerase stability in the reaction conditions

As previously reported, Velaglucerase alfa shows instability issues and loss of enzymatic activity because of environmental and experimental conditions during encapsulation processes (Martinez, 2016). In this scenario, a preliminary work demands the systematic study of the stability of Velaglucerase during the different steps in the conjugation process, in order to quantify the protein degradation or enzymatic activity loss attributable to the technique conditions instead of the conjugation itself.

Although Velaglucerase Alfa, purchased as a freeze-dried powder (Shire Pharmaceuticals), is normally resuspended by adding 4 ml of sterile, MilliQ water (per vial of 10 mg of protein) as indicated in the information pamphlet. In this conditions, the concentration of Velaglucerase is 2.5 mg/ml in Velaglucerase Buffer (VCB), and the pH is 6. However, in order to perform SATP modification, the reaction should be performed over pH 7 (Talelli and Vicent, 2014). Thus, it is necessary a buffer change previously to the SATP modification of the protein. For this, Velaglucerase was diluted at 0.5 mg/ml and its buffer changed to PBS pH 7.4 or VCB pH 6 using a 10 kDa MWCO Amicon in order to test its enzymatic activity at different times and monitor activity loss at different pH. In order to fit in the sensitivity range of the GCase activity it was necessary to dilute the initial solution 1:4000 in VCB. In this conditions, all the samples were virtually at the same pH and ionic conditions for activity measurement. GCase activity was

monitored during 30 hours by a GCase activity assay and the results comparing different pH are shown in figure 4.3).

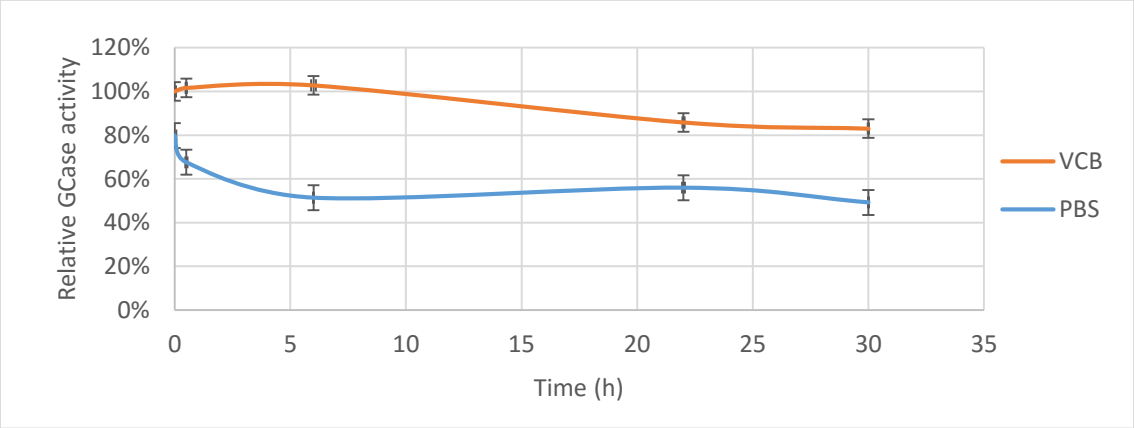

Figure 4.3. Determination of GCase activity of Velaglucerase over time. GCase activity of Velaglucerase alfa monitored in both buffers VCB (orange) and PBS (blue) during 30 hours, at room temperature. The concentration of Velaglucerase was 0.5 mg/ml and the activity is showed as a percentage of Velaglucerase activity at time 0 in VCB. n=3.

These results showed the stability of Velaglucerase with virtually no activity loss for the first 10 hours at room temperature (RT) when VCB buffer is used. For longer times, the stability decreases slowly and only a 20% of activity is lost at 30 hours. As observed, Velaglucerase show less stability in PBS, since the enzyme loss almost 20% of its activity in a few minutes and decreases notably in the first 6 hours. Then, the enzyme retains a 50% of the initial activity during the following hours. As the modification of the enzyme with SATP implies the use of pH higher that 7, we assume an initial loss of activity.

2.2 Modification of Velaglucerase surface with SATP

SATP is the N-hydroxysuccinimide (NHS) ester of S-Acetylthiopropionic acid. NHS functional group reacts with primary amines to generate a stable covalent amide bond. Through SATP modification, a protected sulfhydryl group is added onto the protein surface by reacting with exposed amino groups from lysine side chains (ε-amino group) present in the protein surface. One of the main advantage of this method is the possibility to modify any protein, that has accessible lysines on the surface, with a molecule that contains a protected thiol group which can be deprotected using a deacetylation agent (Talelli and Vicent, 2014). This reaction is pH dependant and is recommended to be performed in a pH range between 7 and 9, and thus PBS at pH 7.4 was used as a buffer for this reaction.

The partial modification of the Velaglucerase surface provokes an inherent non-reversible protein activity loss. This decreasing in activity can be due to the low stability of the enzyme during reaction because the pH 7.4 in PBS or by the functionalization of the enzyme surface with SATP (Talelli and Vicent, 2014; Verheyen et al., 2010). SATP modification implies a non-reversible modification of some of the lysines exposed on the protein surface, so that may alter the local environment of the protein surface as it can disrupt chemical interactions or modify the local charge density and therefore alter the protein activity. In basis of this assumption, we assume that any enzymatic activity lost due to the SATP modification process cannot be later recovered.

Velaglucerase alfa, as well as wild type human glucocerebrosidase, has 22 lysines in its sequence. 20 of them are on the surface of the protein with their ε-amino group totally or partially exposed (2WKL, Protein Data Bank), although none of them takes part of the active site (Brumshtein et al., 2010), as it can be appreciated in Figure 4.4. As curiosity, it is known that mutations in 6 of these lysines are reported to be involved in Gaucher Disease (P04062, UniProt), one of them causing a mild phenotype of GD (K79N) and three of them a severe phenotype (K157Q, K198E and K464E). The K79N mutations is reported to cause GCase a 92% of activity loss, and K198E a complete loss of activity (Liou et al., 2006; Orvisky et al., 2002; Koprivica et al., 2000; Ida et al., 1997). SATP modification over lysines is random, being impossible to control which lysines will be altered. Except for K157 and K186, whose ε-amino groups are not exposed onto the surface, SATP modification can randomly affect lysines that have an effect in the decreasing of GCase activity. The more lysines are affected, the higher is the chance that a critical lysine is affected, and the lower will be the protein activity.

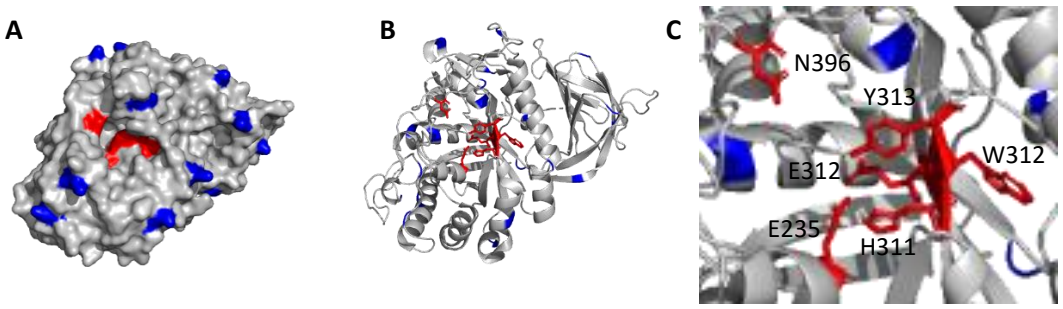

Figure 4.4. Structural representation of Velaglucerase alfa. PDB entry 2WKL, Velaglucerase alfa, was analysed and processed with Pymol 2.1.1. (A) Surface representation of Velaglucerase alfa. Lysine residues are highlighted in blue, residues from the active site are highlighted in red. (B) Ribbon representation of Velaglucerase alfa (lysine residues in blue, residues from the active site in red). (C) Active site of Velaglucerase alfa in a partial view of a ribbon representation. Residues that are part of the active site are highlighted in red and labelled.

In order to minimize the loss of activity during the SATP modification, two key factors needed to be controlled: the SATP/Velaglucerase ratio and the minimum reaction time needed to generate enough SATP functional groups on the protein surface for the PGA-PD coupling in the next step, assuring a minimum decrease in enzymatic activity. Talelli and Vicent (Talelli and Vicent, 2014) tested different molar ratios of SATP/Lysozyme in their work, based on the number of lysines in the protein (6 lysines in a 14.4 kDa polypeptide chain). The SATP/Lysozyme molar ratio for SATP modification was tested at 6 (equimolar ration for lysines contain) and 10 (excess of SATP). For Velaglucerase, three SATP/VPRIP molar ratios were tested considering that there are 22 lysines in Velaglucerase. Thus, ratios of 10, 25 and 40 molar equivalents of SATP were tested in the early stages of the procedure optimization.

In a typical experiment, velaglucerase was added to a PBS solution (pH 7.4) at 0.5 mg/ml, and incubated with 10, 25 or 40 molar equivalents of SATP (in DMSO 20 mg/ml) for 1 hour under agitation at 150 rpm. Early attempts of modification with SATP showed Velaglucerase's propensity to aggregate in presence of moderate amounts of DMSO or under energic agitation (data not shown). After SATP modification reaction, the resultant GBA-SATP is quickly washed with VCB to restore the pH 6, stop the reaction with SATP and minimize the degreasing of GCase activity after the reaction due to the pH. To evaluate the loss of the initial enzymatic activity, different activity assays were performed with the resulting Velaglucerase modified samples. Interestingly, the enzymatic activity decreases notably with the increase of SATP equivalents used in the modification step, as appreciated in figure 4.5. This fact corroborate that the more modification of protein surface, the more enzymatic activity of the enzyme is affected. The higher the SATP/VPRIP rate, the more lysines are modified, and the more GCase activity is lost. When modified with 10 molar equivalents of SATP, Velaglucerase lost around 40% of its activity; with 25 molar equivalents lost up to 2/3 of its activity; and after modification with 40 molar equivalents almost a 90% of the initial activity was lost. The success and optimization of the SATP modification was verified by subsequent conjugation of the modified Velaglucerase with PGA-PD and studies of enzymatic activity recovering after Velaglucerase release in reducing conditions, as described in the next section.

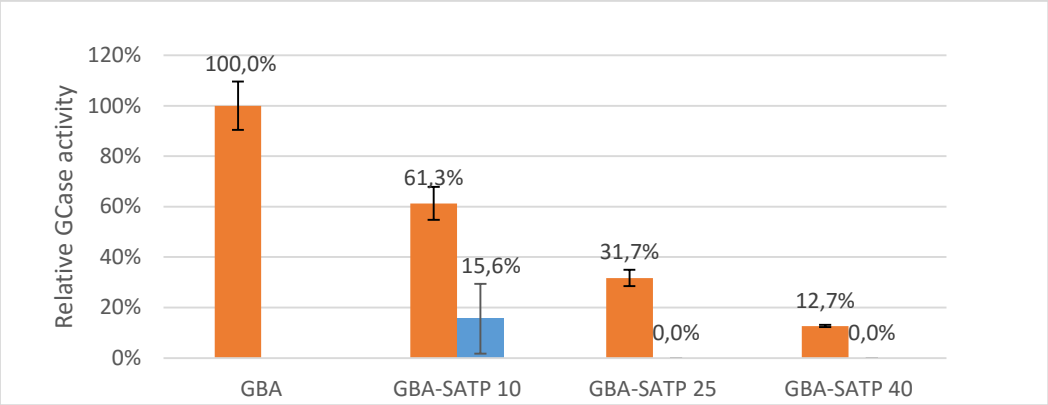

Figure 4.5. GCase activity of Velaglucerase throughout the conjugation process. Relative GCase activity of Velaglucerase alfa before and after modification of the surface with SATP using different concentrations (10, 25, 40 equivalent) (in orange). GCase relative activity of unmodified GBA is considered 100%. The recovered activity of GBA-PGA conjugates after reaction with GSH 10 mM for 2 hours only was appreciated in the case of the nanoconjugate modified with 10 equivalents of SATP (in blue).

2.3 Velaglucerase conjugation with PGA

After modification of Velaglucerase with different concentrations of SATP, the resultant GBA-SATP samples were exposed to 5 molar equivalents of PGA-PD (excess) in presence of a deacetylation solution overnight. The deacetylation solution deprotects the thiol groups added by SATP modification, allowing the PGA-PD to form disulphide bonds with them and attach covalently to protein surface.

After the conjugation, Velaglucerase-PGA samples were washed with VCB several times and were analysed by size exclusion chromatography (SEC) by means of fast protein liquid chromatography (FPLC). The results, showed in figure 4.6, allow the estimation of the number of PGA chains per protein based in the samples elution time. From the elution profile, it has been calculated that conjugates from Velaglucerase modified with 10 molar equivalents of SATP contained 3 chains of PGA per Velaglucerase molecule, while conjugates from Velaglucerase modified with either 25 or 40 molar equivalents of SATP, contained between 4 or 5 chains of PGA per Velaglucerase molecule. Velaglucerase and PGA were also analysed by SEC as a control (data not shown), so that the number of PGA chains per conjugate could be estimated.

This method allow to know the maximum number of PGA chains that can attach to the protein surface. Interestingly, although the protein surface is functionalized with different number of

SATP molecules the maximum number of PGA molecules that can incorporate per Velaglucerase unit is 5. In fact the reaction of the Velaglucerase-SATP samples obtained with different concentration of SATP give the same results if they react with 5 equivalents of PGA-PD. The enzymatic activity assays using GBA-PGA samples indicated that in all cases no activity was detected. This fact would indicate that the PGA chains effectively coat the protein surface and protects it form the environment, including access towards the active site. Thus, the GBA activity will be masked until the PGA coating is eliminated from the enzyme surface. To test the final recovered activity of Velaglucerase after SATP modification, PGA conjugation and release, a simulated reductive environment similar to this found in the lysosome, the conjugates were dispersed in VCB with 10 mM of GSH, for 2 hours at 37ºC. As observed in figure 4.5, only the recovering of enzymatic activity was observed for the Velaglucerase modified with 10 molar equivalents of SATP, meanwhile negligible activity was recovered in the case of the more modified enzyme. These results indicates that the anchoring of 3 chains of PGA, observed in the conjugates modified with 10 molar equivalents of SATP, may be enough for successful GCase activity masking, and for recovering notable activity levels after PGA unmasking process in reducing environment. The fact that we cannot recovered the initial enzymatic activity observed before PGA conjugation makes us think that probably part of the enzymatic activity is lost in the process of conjugation or there is not a complete elimination of PGA coating by reduction of disulphide bonds.

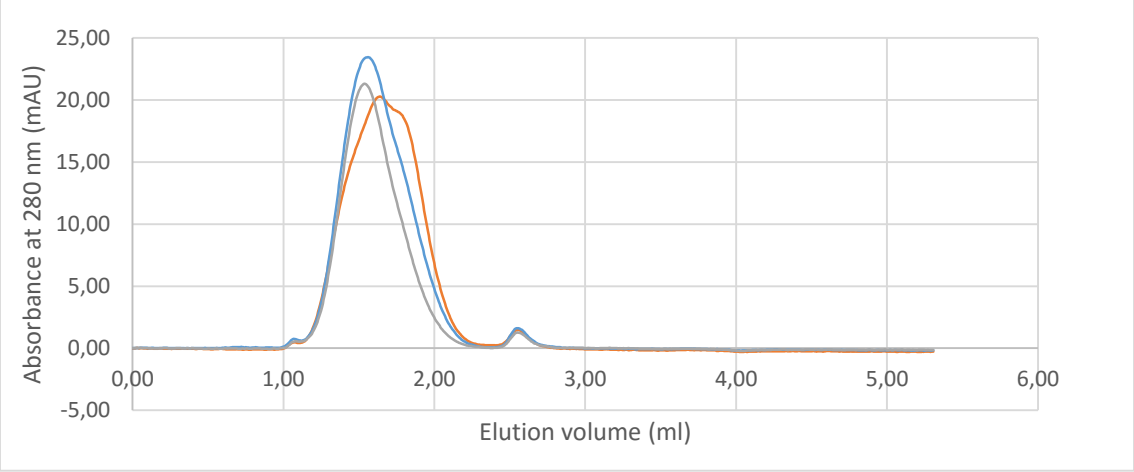

Figure 4.6. Size exclusion chromatography of the GBA-PGA conjugates. Size exclusion chromatograms by means of FPLC with protein detection at 280 nm.. Conjugates analysed were synthesised after modification with 10 molar equivalents of SATP (orange), 25 molar equivalents (blue) and 40 molar equivalents (grey).

At this point we consider that the nanoconjugate obtained after modification of the protein surface with 10 equivalents and subsequent conjugation of PGA, is able to keep mask the enzymatic activity of Velaglucerase and recover part of its activity once the PGA coating is eliminated in reducing environments. Once the nanoconjugate synthesis was optimized, the next step consisted in evaluate *in vitro* the capacity of these modified enzymes to target the lysosome and restore de GCase activity in GBA knockout cells after cell internalization. The reducing environment and pH of Lysosome should be adequate for the Velaglucerase release in its native and active form.

2.4 Conjugate internalization in neuron cells and activity restoration. Early stages.

Different attempts to restore the Wild Type (WT) phenotype in GBA knockout neuroblastoma cells were performed using the optimized GBA-PGA conjugates. The studies consisted on testing the *in vitro* internalization of GBA-PGA conjugates in a GBA knockout BE(2)-M17 cell line, their intracellular localization, the delivery of Velaglucerase into the lysosome and the measurement of activity restoration. As previously observed (Martínez, 2016), a 3-hours exposition of PLGA-encapsulated Velaglucerase was enough for the protein to reach the lysosome. Taking into account this study, we decide test the behaviour of GBA-PGA nanoconjugates and evaluate the restoration of GCase activity in comparison with the administration of non-modified Velaglucerase. For this, four conditions were studied: 1) BE(2)-M17 WT cells were kept untreated as a positive control for GBA content and activity in a WT phenotype, 2) M17 GBA-KO cells were kept untreated as a negative control for GBA content and activity in knockout cells, 3) M17 GBA-KO cells were treated for 3 hours with 25 µg/ml of Velaglucerase alfa and 4) M17 GBA-KO cells were treated for 3 hours with 25 µg/ml of a GBA-PGA conjugate. Treated cells were collected and processed for subcellular fractionation as described in Experimental procedures. Velaglucerase content of each fraction was analysed by western blot and a GBA activity assays for the cells treated with Velaglucerase and GBA-PGA were performed.

The quantification of GBA by Western blot after subcellular fractionation and GCase activity assay tested for the GBA present in homogenate, cytosol and lysosomes was performed and represented in figure 4.7. As observed, both the GBA-PGA conjugates and Velaglucerase are internalized into the lysosome, although internalization levels are significantly higher in unmodified Velaglucerase (figure 4.7a). However, no significant increase in GBA activity was

detected in the different fractions after conjugate treatment while cells treated with Velaglucerase did show a GBA activity increase (figure 4.7b).

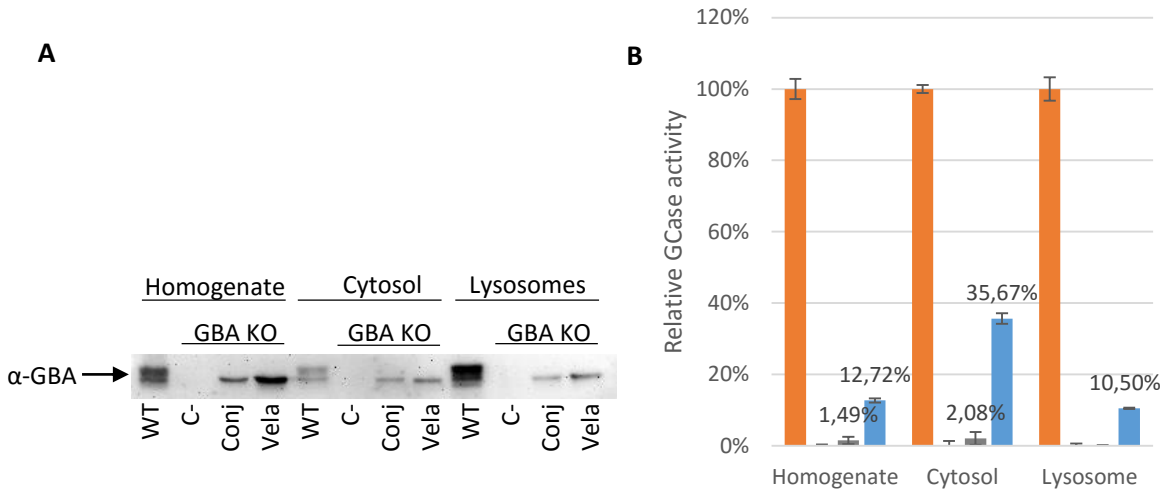

Figure 4.7. *In vitro* treatment of M17 cells with Velaglucerase conjugates. M17 were treated for 3 hours with 25 μg/ml of conjugate prepared from Velaglucerase modified with 10 molar equivalents of SATP. Four conditions were established: untreated WT M17 cells (WT, orange), untreated GBA knockout M17 cells (C-), GBA knockout M17 cells treated with conjugate for 3 hours (Conj, blue) and GBA knockout M17 cells treated with unmodified Velaglucerase for 3 hours (Vela, grey). (A) Western blot with anti-α-GBA antibody after subcellular fractionation and isolation of the lysosomal fraction. (B) GCase activity assay (same samples from A). Relative GCase activity was calculated; in each fraction WT was set as 100% and C- as 0%.

At this point, a sensible optimization of the conjugation protocol was necessary, in order to minimize the GCase activity loss so that the conjugates retain enough levels of activity to restore GCase activity into the GBA knockout cells. The most sensitive parameters considered for optimization were those in which we consider that the loss of initial activity is more critical based in the previous results: SATP modification and PGA-PD conjugation times. We assume, as Velaglucerase is more stable in VCB, a buffer at pH 6, than is in PBS (Figure 4.3)that the longer time the enzyme is at a pH different from pH 6, the greater the loss of non-recoverable activity.

2.5 Time optimization of Velaglucerase conjugation in VCB

For the optimization of the reaction time during the Velaglucerase conjugation different assays were performed to minimize the time necessary to obtain the conjugate. Thus GBA-SATP at 0.5 mg/ml in VCB pH 6 buffer was incubated with 5 molar equivalents of PGA-PD in presence of deacetylation buffer. Conjugation progression with time was qualitatively assessed by non-reducing SDS-PAGE of the conjugate developed with silver staining (Figure 4.8A). The PGA/GBA ratio were calculated in all the cases by Size Exclusion Chromatography (SEC) to corroborate the optimized coating of the protein (Figure 4.8B).

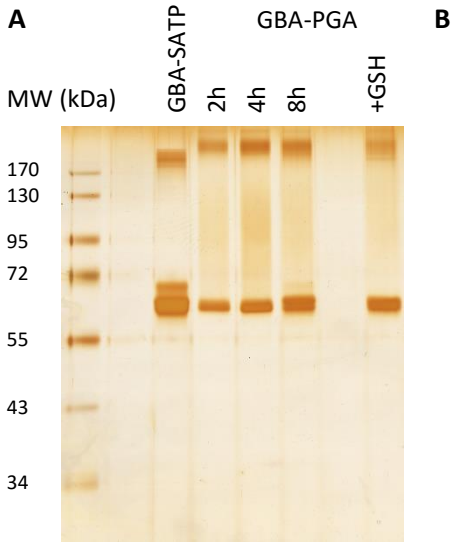

Figure 4.8. Conjugation of GBA-PGA. Velaglucerase modified with 10 molar equivalents of SATP were incubated with PGA-PD in presence of deacetylation buffer and samples were taken at 2, 4 and 8 hours. Release of GBA-SATP was performed on the GBA-PGA obtained after 4 hours of conjugation, with GSH 20 mM in VCB. (A) Non-reducing SDS-PAGE was run to qualitatively assess the conjugation progress and developed with silver staining. (B) SEC

As observed in Figure 4.8 (A) there is a qualitative progression of the conjugation with time. When conjugated, Velaglucerase experience an increase in molecular weight due to the addition of PGA chains, which can be detected by electrophoresis. The evolution in the conjugation observed by SEC (4.8 B) indicate that the complete conjugation (3 chains of PGA per protein) is obtained at 4 hours. Below this time, incomplete conjugation was found and no more than 3 chains per protein was achieved for long time.

2.6 Conjugate internalization in neuron cells. Optimized conjugates

After the optimization of the conjugation procedure, a freshly prepared batch of GBA-PGA conjugates was tested in M17 GBA knockout cells to assess its capacity to restore GCase activity in the intracellular environment. As previously, four conditions were tested: 1) BE(2)-M17 WT cells were kept untreated as a positive control for GBA content and activity in a WT phenotype, 2) M17 GBA-KO cells were kept untreated as a negative control for GBA content and activity in knockout cells, 3) M17 GBA-KO cells were treated for 3 hours with 6 µg/ml of Velaglucerase alfa and 4) M17 GBA-KO cells were treated for 3 hours with 6 µg/ml of a GBA-PGA conjugate.

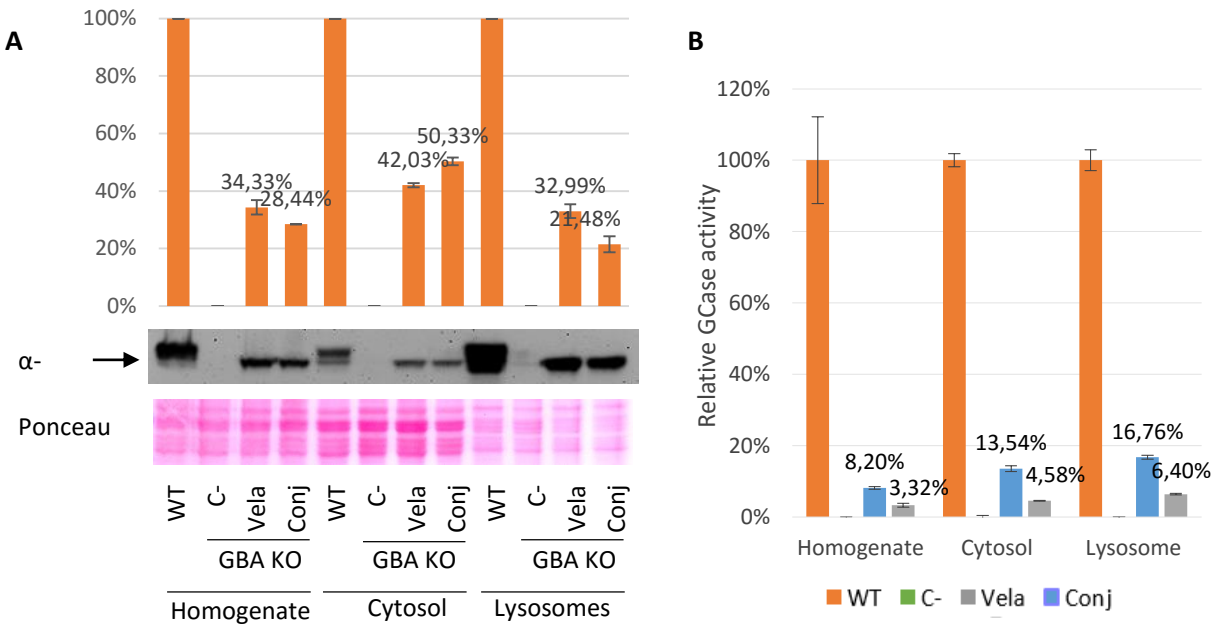

Figure 4.9. ***In vitro*** **treatment of M17 cells with Velaglucerase conjugates.** M17 cells were treated for 3 hours with 5 µg/ml of Velaglucerase. Four conditions were established: untreated WT M17 cells (WT, orange), untreated GBA knockout M17 cells (C-), GBA knockout M17 cells treated with unmodified Velaglucerase for 3 hours (Vela, blue) and GBA knockout M17 cells treated with a GBA-PGA conjugate for 3 hours (Conj, grey). (A) Western blot with anti-α-GBA antibody was done after subcellular fractionation and isolation of the lysosomal fraction. . Relative GBA levels were calculated for each fraction (WT was set as 100% and C- as 0%). (B) GCase activity assay (same samples from A).. Relative GCase activity was calculated; in each fraction WT was set as 100% and C- as 0%.

Many interesting data can be extracted from Figure 4.9. First of all, we can observe that the GBA-PGA conjugates, after 3 hours exposure at 5 µg/ml on M17 GBA knockout cells, are able to partially restore GCase activity. These results corroborate that the optimization of the

reaction parameters to coat the protein has preserve a part of the enzymatic activity and it is a step forward compared with the results obtained before the conjugate synthesis optimization. After treating cells with the same Velaglucerase concentration, either conjugated or not, unmodified Velaglucerase restores an 8.20% of the WT activity, and the conjugates a 3.32% which is about a 40% of the activity restoration if we compare with non-modified enzyme. Inside the lysosomes, the relative GCase activity recovered by both non-conjugate Velaglucerase and the conjugate is higher than in the homogenate, as Velaglucerase recovers a 16.76% of the WT activity and the conjugate a 6.4% (also around a 40% of the activity recovered by unmodified Velaglucerase).

However, it's important to notice that, despite the cells are treated with the same concentration of protein, unmodified Velaglucerase reaches higher levels of internalization, globally and in the lysosome. The conjugates, on the other hand, show less cellular internalization, less localization in the lysosome and more permanence in the cytosol than unmodified Velaglucerase. This means that the conjugates have a hindered internalization both into the cell and into the lysosome. Being observed that the activity levels of cells treated with Velaglucerase and the conjugates were lower than those in the WT, but protein levels were also lower, activity levels were normalized with the amount of Velaglucerase from each sample.

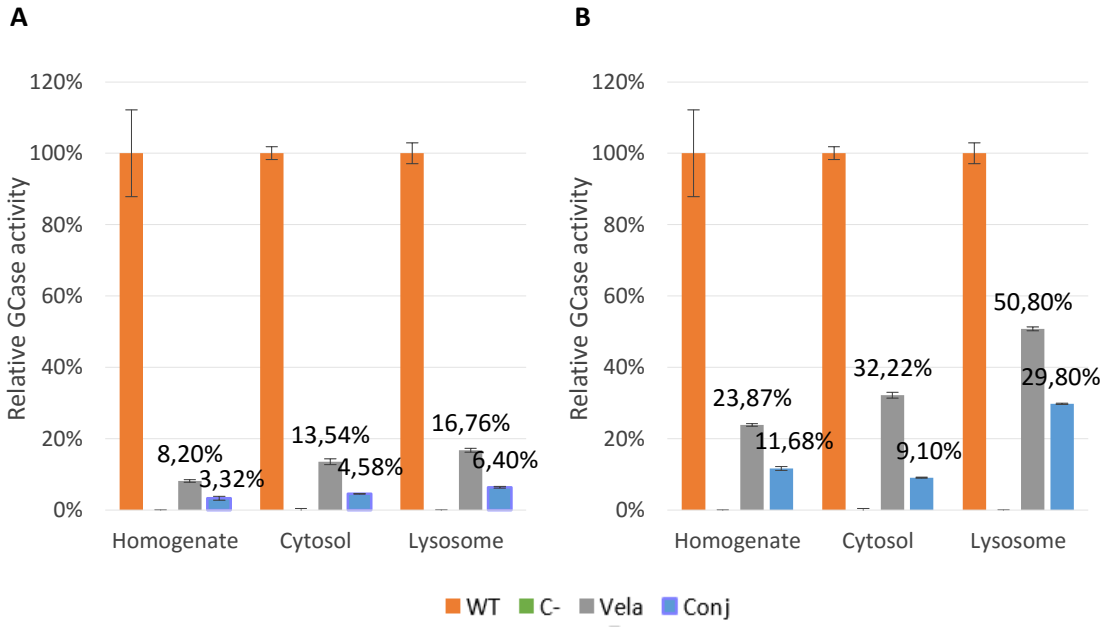

Figure 4.10. Normalization of GCase activity levels. Comparison of (A) GCase activity normalized with the GBA levels of each sample, calculated by densitometry of Western Blot, and (B) Relative GCase activity after normalization.

Relative GCase activity after normalization (Figure 4.10) shows the theoretical activity that would be restored if the levels of internalized Velaglucerase in the lysosome reached the amount of GCase from WT cells. It seems therefore, that Velaglucerase, once exposed to M17 cells, internalized and localized in the lysosome, keeps around 50% of the native activity. Conjugated Velaglucerase, in the same conditions and once internalized and localized in the lysosome, keeps around 30% of the native activity. If we compare directly the conjugates with unmodified Velaglucerase, around 60% is recovery.

2.7 Test of conjugates' protective ability

As mention in the introduction of this chapter, one of the main drawbacks of using Velaglucerase for ERT treatments is the extremely low stability of the enzyme in physiological conditions and by extension, in the bloodstream. It is a critical problem for the administration of Velaglucerase since most of the active enzyme is lost during the transit until reaching the site of action. In order to probe the protective ability of the PGA coating in physiological conditions, specifically in blood, different assays were performed to check the stability of the nanoconjugate in human blood plasma. Our hypothesis is that if conjugates are protective from the harsh conditions of the bloodstream, when incubating Velaglucerase and GBA-PGA conjugates in human blood plasma we should see a decay in the GCase activity in unmodified Velaglucerase, while conjugates should slow down or even impede the activity loss. This experiment would corroborate the suitability of this kind of methodology for protecting Velaglucerase during the permanence in bloodstream as inactive form, until the conjugate reach the lysosomes and the reductive conditions inside lysosome activate the enzymatic action.

For assessing this behaviour, Velaglucerase and GBA-PGA conjugates were incubated for 1 and 3 hours in human blood plasma prior to the treatment of M17 GBA knockout cells for 3 hours with 6 μg/ml of conjugated or non-modified protein. After 3 hours, treated cells were collected and a subcellular fractionation was performed. Samples were analysed by α-GBA western blot and GCAse activity assay were performed. Results are shown in Figure 4.11.

As observed in the western blot analysis for the subcellular fractionation (Figure 4.11A) the distribution of the enzyme follow the same pattern that previously observed. The highest concentration of GBA is in the lysosome and the amount of non-modified Velaglucerase is

higher than the corresponding GBA-PGA. The levels of GBA internalised are far from the native levels of GBA in the WT cells, and Velaglucerase shows a better internalization than the conjugates. However, signs of protein degradation can be observed in all the plasma-incubated samples, especially the unmodified Velaglucerase that shows the more intense degradation bands. However, the activity test indicate very interesting results (Figure 4.11B). In these assays low enzymatic activity was detected in homogenate and cytosol due to the presence of low amounts of GCase (in some cases under the detection limit). In the homogenate only non-modified Velaglucerase incubated 1 hour in plasma shows enough Gcase activity to be detected, and in the cytosolic fraction only the samples incubated in plasma for 1 hour show enough activity to be detected. However, the most interesting result was observed in the lysosomal fraction. Although after 1 hour of incubation in plasma the comparative activity of non-modified Velaglucerase is clearly superior to the nanoconjugate, when the incubation in plasma takes 3 hours the situation reverses in a remarkable way and now the GCase activity of the unmodified falls drastically until it is almost not detected while much more activity is obtained for the conjugates. When GCase activity is normalised with the protein contain, high levels of GCase activity recovery can be appreciated specially in the lysosomal fraction. Cells treated with Velaglucerase incubated for 1 hour in plasma, if the levels of GBA reached those in the WT cells, would recover around 90% of the native activity, while those cells treated with GBA-PGA conjugates incubated in plasma for 1 hour would recover around half of the native activity. It is interesting to see that after being the proteins incubated for 3 hours in plasma, cells treated with Velaglucerase would show almost no GCase activity, while cells treated with conjugates would show a noteworthy activity recovery around a third of the native GCase activity. All these results corroborate the protective effect of the PGA coating on Velaglucerase and validate this methodology as an alternative mode to protect this kind of high sensitive enzymes in physiological environments until the active specie reach the site of action.

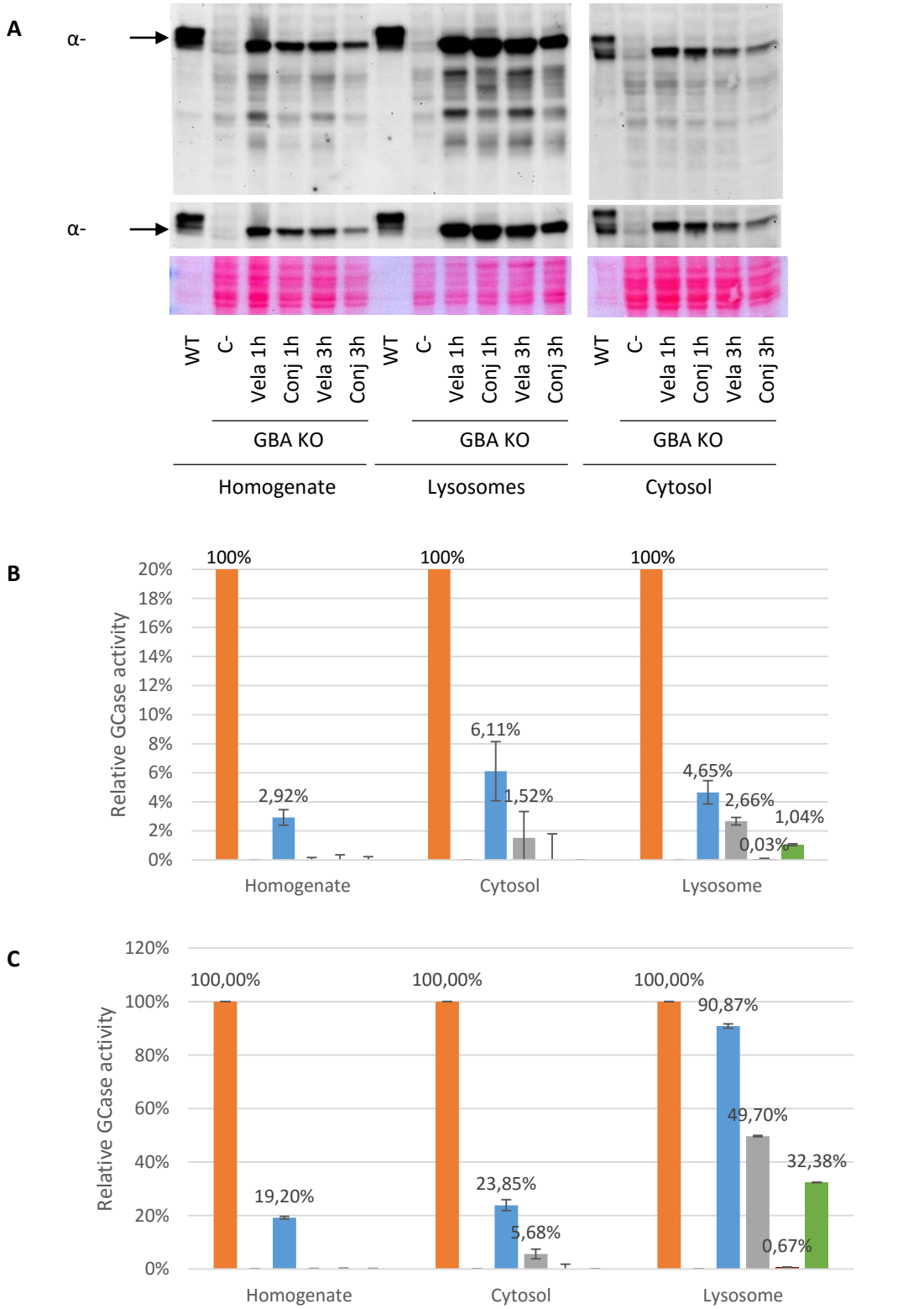

Figure 4.11. ***In vitro*** **treatment of M17 cells with plasma pre-incubated Velaglucerase conjugates.** Velagluceraase and GBA-PGA conjugates were incubated for 1 and 3 hours in human plasma before treating M17 GBA knockout cells for 3 hours. Six conditions were established: untreated WT M17 cells (WT, orange), untreated GBA knockout M17 cells (C-), GBA knockout M17 cells treated for 3 hours with unmodified Velaglucerase previously incubated in human plasma for 1 hour (Vela 1h, blue), GBA knockout M17 cells treated for 3 hour with a GBA-PGA conjugate previously incubated in human plasma

for 1 hour (Conj 1h, grey), GBA knockout M17 cells treated for 3 hours with unmodified Velaglucerase previously incubated in human plasma for 3 hours (Vela 3h, red) and GBA knockout M17 cells treated for 3 hour with a GBA-PGA conjugate previously incubated in human plasma for 3 hours (Conj 3h, green). (A) Western blot with anti-α-GBA antibody after subcellular fractionation and isolation of the lysosomal fraction. Relative GBA levels were calculated for each fraction (WT was set as 100% and C- as 0%). (B) GCase activity assay:; in each fraction WT was set as 100% and C- as 0% (samples under detection limit are shown as 0). Due to the low percentage of GCase activity in most of the samples, the Y-axis only shows values between 0% to 20%. (C) Relative GCase activity after normalization: activity levels were normalized with the GBA levels of each sample, calculated by densitometry from the western blot (Figure 4.11A).

General discussion

GENERAL DISCUSSION

In this thesis, we explored different nanotechnological strategies in order to improve the therapeutic properties of proteins with clinical applications by protecting them against harmful environmental conditions, prolonging their half-life and increasing their bioavailability. Depending on the nature of each protein, regarding size, structural complexity and enzymatic activity amongst other physicochemical properties; the strategies on each protein may vary greatly.

The first group of proteins addressed in this thesis are the Epidermal Growth Factor (EGF) and the *Nerita versicolor* Carboxipeptidase Inhibitor (NvCI). Both proteins have in common their small size, around 6 kDa, its simple structure due to its low molecular weight, a similar isoelectric point and a non-enzymatic biologic activity. Their robustness conferred by their simple conformation and the presence of disulphide bonds made them good candidates for encapsulation in PLGA nanoparticles, as synthesis conditions shouldn't be critically harmful for their biological activity. The mitogenic activity of EGF was of interest as many studies describe its ability to increase the healing rate of peptic ulcers in the gastrointestinal lumen. However, EGF is susceptible to degradation and subsequent loss of activity by proteases, abundant in the gastrointestinal environment. Coencapsulation of EGF with NvCI was designed in order to protect EGF from degradation by the digestive metallocarboxipeptidases (MCPs).

The protective effect of NvCI was proved when we exposed EGF to MCPs, which cleaved the C-terminal tail of EGF, transforming it into less active or unactive forms of EGF. MCPs were successfully inhibited when incubation of EGF was performed in presence of NvCI. Co-encapsulation of both proteins in PLGA nanoparticles should deliver them together to their target and thus protecting EGF from C-terminal protease degradation while performing its biological activity.

EGF and NvCI were successfully encapsulated in PLGA nanoparticles, separately and in co-encapsulation, after a thorough process of synthesis optimization. Reproducible, spherical and monodisperse nanoparticles were obtained, with EGF and NvCI encapsulation efficiency also reproducible, around 40% either when encapsulated separately or coencapsulated. Furthermore, after encapsulation both proteins maintained their biological activity.

Gastric simulation assays to assess NvCI protective effect upon release in the environmental conditions found in the gastrointestinal lumen were unsuccessful, as no protein release nor

biological activity could be detected at the time the gastric simulation finished, neither in the controls. One explanation could be related with the fact that PLGA release may be too in the 5 hours that gastric simulation lasts or perhaps the gastric conditions are too strong for PLGA nanoparticles.

The second protein addressed by this thesis for protein encapsulation was Velaglucerase alfa. Velaglucerase is a recombinant version of the human β-glucocerebrosidase, used for Enzyme Replacement Therapy (ERT) in Gaucher Disease. Many important differences arise between Velaglucerase and EGF and NvCI. Velaglcerase is a much larger protein, of 55.6 kDa, with intrinsic enzymatic activity and a complex and fragile structure. Its poor stability and low residence time in the bloodstream after parenteral administration and its inability to cross the BBB made Velaglucerase a good candidate for a therapeutic improvement by means of a nanotechnological strategy, as protein stability could be improved and further functionalization could allow it to cross the BBB and be able the treatment of the neurological effects of Gaucher Disease.

Early attempts to encapsulate Velaglucerase in PLGA nanoparticles failed as, even after many efforts to optimize the synthesis procedure, the enzymatic activity of Velaglucerase was completely lost.

Another strategy for Velaglucerase needed to be planned, a strategy that didn't compromise protein stability and enzymatic activity but allows the nanocosntruct to reach the lysosomes and release Velaglucerase maintaining its activity.

Polymer Masked-Unmasked Protein Therapy (PUMPT) was presented as a promising alternative to PLGA encapsulation, as it consisted in the conjugation of a protein (Velaglucerase) with the non-toxic, non-immunogenic and biocompatible Poly(L-Glutacic acid) (PGA) polymer. After a thorough optimization of the experimental parameters for the conjugation process in order to minimize the decreasing of enzymatic activity, Velaglucerase was successfully conjugated with PGA. Conjugates completely masked the enzymatic activity of the protein.

Internalization assays in neuroblastoma cells showed not only that the conjugates were able to reach the lysosomes after internalization but also Velaglucerase was released and maintained about 60% of its original activity. The conjugates internalization, however, is slightly inferior than internalization of unconjugated Velaglucerase.

In order to assess the protective capacity of the conjugation to protect coated Velaglucerase against environmental conditions that may decrease its enzymatic activity, the internalization assays were repeated after pre-incubation of the conjugates and of unconjugated Velaglucerase in human blood plasma, for different periods of time. Although unconjugated Velaglucerase s able to maintain most of its activity after short exposure times, after three hours of incubation it suffers a dramatic, almost complete loss of activity, while Velaglucerase released from the conjugates manages to maintain a third of the native GBA activity.

Concluding Remarks

CONLUDING REMARKS

Design of a therapy based in the encapsulation of EGF for treatment of Peptic Ulcers.

1. Synthesis parameters of PLGA nanoparticles were successfully optimized for the co-encapsulation of NvCI and EGF, obtaining reproducible, spherical, monodisperse nanocapsules. EGF and NvCI were encapsulated essentially in an equimolar manner.

2. Encapsulation efficiency (EE) of EGF and NvCI was found reproducible, with values around 40%. The EE values for EGF and NvCI showed no significant differences when encapsulated separately or when co-encapsulated.

3. After encapsulation, both EGF and NvCI were qualitatively proven to retain its biological activity. EGF extracted from PLGA nanoparticles was able to induce EGFR phosphorylation in A431 cells, and NvCI extracted from nanoparticles maintained its inhibitory activity on digestive carboxiopeptidases.

4. Digestive carboxipeptidases (CPs) are shown to cleave the C-terminal tail of EGF, which results in a decrease of its mitogenic activity. NvCI is proven to effectively protect EGF from degradation after at least 2 hours of exposure to digestive CPs. EGF in presence of NvCI maintains a functional C-terminal and thus retains its biological activity.

5. The suggested approach for gastric simulation was unable to observe an effect of EGF in a wound healing rate assay. However, scratch assay technique was successfully set up and was responsive to EGF stimulation.

1. Encapsulation methods in PLGA nanocapsules were found incompatible for Velaglucerase alfa, as its structure and thus its enzymatic activity was irreversibly damaged. Many optimization steps were performed to increase Velaglucerase stability in PLGA nanocapsules, all of them unsuccessful.

2. Bioinformatics studies on Velaglucerase structure showed that this enzyme presents 22 lysines in its primary structure, 20 of them accessible in the surface and susceptible to be modified. The study of the active site reveals that no lysine residues were involved in the catalysis. However, at least 6 lysines are reported in the bibliography that are involved in Gaucher disease when mutated, affecting the enzymatic activity.

3. A conjugation method based on Polymer Masked-Unmasked Protein Therapy (PUMPT) was successfully adapted for Velaglucerase, using the biocompatible, amino acid-made polymer Poly(L-glutamic acid) (PGA).

4. Velaglucerase-PGA conjugation procedure was optimized in terms of pH, buffer composition, reaction times, storage conditions and the number of lysines susceptible to be modified and thus to minimize the enzymatic activity loss.

5. Enzymatic activity of Velaglucerase was successfully, and completely masked after PGA conjugation. Intracellular release of Velaglucerase after internalisation assays showed that almost 60% of the enzymatic activity was maintained after conjugation, and thus nanoconjugates were able to partially restore GCase activity in GBA KO cells.

6. Pre-incubations of the nanoconjugates with human blood plasma showed an improvement of Velaglucerase stability when conjugated, as after 3 hours of incubation, unconjugated Velaglucerase had lost more than 99% of its activity, while conjugated Velaglucerase retained almost a third of it.

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