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# IS THERE AN IMPACT OF ENCAPSULATION ON PARENTERAL DELIVERY OF INSULIN?

Dissertation of Pharmaceutical Biotechnology Master Degree under orientation of Professor Ph.D. António José Ribeiro, presented to the Faculty of Pharmacy of University of Coimbra.

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# Is there an impact of encapsulation on parenteral delivery of insulin?

Dissertation of Pharmaceutical Biotechnology Master Degree presented to the Faculty of Pharmacy, University of Coimbra to obtain the degree of Master

Oriented by: Professor Ph.D. António José Ribeiro

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## ABSTRACT

Diabetes *mellitus* is a chronic metabolic disorder with epidemic proportions which is characterized by chronic high blood glucose levels as a result of the inefficient insulin action. It is classified in different types, such as type 1 and type 2, and its development depends on several factors, such as lifestyle and genetic factors. For insulin-dependent diabetics the administration of insulin is the only available therapy.

Insulin therapy, as other protein therapies, is challenging due to protein properties, such as susceptibility to physical and chemical degradation and short *in vivo* half-life, and, therefore its delivery has to be made by parenteral route. Since it began, insulin therapy has evolved trying to better mimic the physiological profile of insulin action in healthy individuals. As some limitations still compromise the efficiency of insulin therapy, encapsulation of insulin has been proposed in order to improve its long-term delivery by parenteral route.

In the present thesis a brief review of the evolution of insulin therapy since its beginning will be made. It is intended to identify the main disadvantages of current insulin therapy. Also, the potential application of nanotechnology in insulin delivery will be addressed. The encapsulation of insulin into polymeric particles and its effect in the control of blood glucose levels after parenteral administration will be also discussed.



**Keywords:** insulin, parenteral delivery, polymeric particles, encapsulation

# TABLE OF CONTENTS

*Acknowledgments* ..... *i*

*Abstract* ..... *iii*

*Table of contents* ..... *v*

*List of Abbreviations* ..... *vii*

*List of figures* ..... *ix*

*List of tables* ..... *xi*

*1. Introduction* ..... *1*

1.1. Diabetes Mellitus ..... 1

1.2. Insulin ..... 3

1.3. Insulin Therapy ..... 5

*2. Nanotechnology and Health Care* ..... *11*

2.1. Carrier-based Drug Delivery Systems (carrier-DDS) ..... 11

2.1.1. Polymer-based Carriers ..... 16

2.1.1.1 Types of polymers ..... 17

2.1.1.2. Formulation methods ..... 19

2.1.1.3. Commercialized encapsulated peptides/proteins ..... 21

*3. Parenteral administration of encapsulated insulin* ..... *23*

*4. Conclusion* ..... *43*

*5. References* ..... *45*

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Adm. | Administration |
| AM | Alloxan Monohydrate |
| Arg | Arginine |
| Asn | Asparagine |
| Asp | Aspartic Acid |
| ATP | Adenosine Triphosphate |
| BB | BioBreeding |
| BGL | Blood Glucose Levels |
| Carrier-DDS | Carrier-based Drug Delivery Systems |
| $Ca^{2+}$ | Calcium Ion |
| $CO_2$ | Carbon Dioxide |
| DIC | Dual Interaction Complex Between Insulin and Methoxy Poly(ethylene glycol)-branched Oligoethylenimine-Poly (L-histidine) |
| DM | Diabetes Mellitus |
| EMA | European Medicines Agency |
| GH | Growth Hormone |
| Glu | Glutamic Acid |
| GLUT 2 | Glucose Transporter Type 2 |
| GLUT 4 | Glucose Transporter Type 4 |
| Gly | Glycine |
| HGC | Hydrophobically Modified Glycol Chitosan |
| IM | Intramuscular |
| IP | Intra-peritoneal |
| IV | Intravenous |
| $K^+$ | Potassium Ion |
| Lys | Lysine |
| MPS | Mononuclear Phagocytic System |
| MP | Microparticle |
| MW | Molecular Weight |
| NIH | National Institute of Health |

| | |
|---|---|
| NPH | Neutral Protamine Hagerdorn |
| NP | Nanoparticle |
| NT | Nanotechnology |
| o/w | Oil-in-Water |
| PBS | Phosphate Buffer Saline |
| PCL | Poly-ε-Capralactone |
| PEG | Polyethylene Glycol |
| PLA | Poly(lactic acid) |
| PLGA | Poly(lactic-co-glycolic acid) |
| PMA | Polymethacrylate |
| PMAA | Poly(methacrylic acid) |
| POH | Methoxy Poly(ethylene glycol)-Branched Oligoethylenimine-Poly (L-histidine) |
| Pro | Proline |
| PVA | Poly(vinyl alcohol) |
| p(CPP:SA) | Poly 1,3-bis-(p-carboxyphenoxy) Propane-co-Sebacic-Acid |
| P(FASA) | Poly(fumaric-co-sebacic anhydride) |
| SA | Sebacic acid |
| SC | Subcutaneous |
| SD | Sprague-Dawley |
| SDS | Sodium Lauryl Sulfate |
| STZ | Streptozotocin |
| s/o/s | Solid-in-Oil-in-Water |
| s/o/w | Solid-in-Oil-in-Water |
| Thr | Threonine |
| T1D | Type 1 Diabetes |
| T2D | Type 2 Diabetes |
| w/o/w | Water-in-Oil-in-Water |

## LIST OF FIGURES

**Figure 1.** Estimated number of people living with DM worldwide in 2015 and 2040

**Figure 2.** Primary human insulin structure

**Figure 3.** Glucose-stimulated insulin secretion by the β-cells of the pancreas of a non-diabetic subject

**Figure 4.** Glucose uptake by a target cell (such as hepatocytes and adipocytes) of a non-diabetic subject

**Figure 5.** Timeline for insulin developments

**Figure 6.** Polymeric micro and nanoparticles

**Figure 7.** Drug concentrations in plasma after multiple administration in a chronic disease

**Figure 8.** Drug concentrations in plasma after administration of a prolonged release formulation

## LIST OF TABLES

**Table 1.** Current insulin analogues commercially available in Europe

**Table 2.** Insulin pharmacokinetic profiles

**Table 3.** Some types of carriers

**Table 4.** Examples of natural and synthetic polymers used as delivery systems

**Table 5.** Some examples of approved peptides/proteins delivered by polymeric carriers

**Table 6.** Physicochemical characterization of insulin-loaded polymeric particles with potential antihyperglycemic activity after parenteral administration

# 1. INTRODUCTION

## 1.1. Diabetes Mellitus

Diabetes *mellitus* (DM) is a chronic metabolic disorder that has reached epidemic proportions worldwide and became a burden in human and financial terms. According to *International Diabetes Federation* (IDF), 415 million people throughout the world were living with DM in 2015 (prevalence: 8.8%) and 46.5% of them were undiagnosed. The higher number of diabetic people was observed in the Western Pacific Region where more than 153 million adults were suffering from DM in the last year. However, the highest prevalence per capita is observed in the North America and Caribbean Region with one out of eight adults living with the disease. Moreover, approximately 5.0 million deaths in 2015 due to DM. Also worthy of mention is that younger and younger people are developing DM which is a worrying trend. Additionally, the number of diabetics is likely to increase in all regions of the world. It is estimated that 642 million people will be living with DM in 2040 (prevalence: 10.4%). As the number of diabetic people increases, the costs to society are increasingly high. In 2015, the global health expenditure due to the treatment of diabetes and associated complications was estimated to range from US$ 673 billion to US$ 1,197 billion. By 2040, it will range from US$802 billion to US$1,452 billion. Therefore, the better understanding of the disease, the early diagnostic, the improvement of DM therapy and the education of the population are of major importance in order to reverse the disturbing trend of increase in the number of diabetic people. (1)

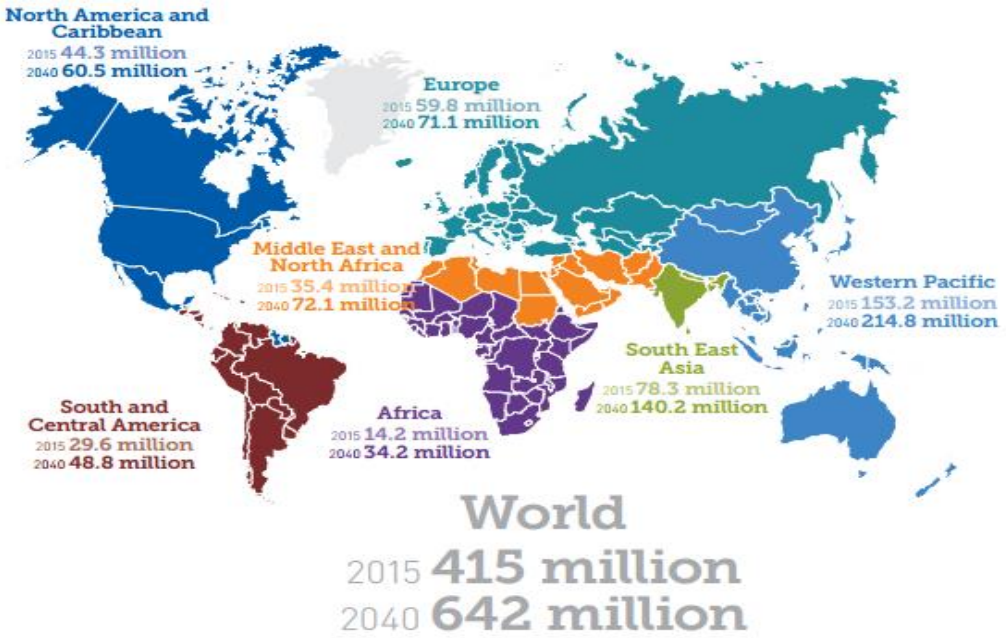


**Figure 1: Estimated number of people living with DM worldwide in 2015 and 2040**. (1)

DM is characterized by chronic hyperglycemia (high blood glucose levels) that results from deficient insulin action. In other words, a total or relative lack of insulin secretion and/or insulin resistance are responsible for the altered glucose metabolism observed in diabetic patients. (2, 3) It is well established that the interaction between genetic and environmental factors are implicated in the development of DM. However, the exact mechanism of development of the disease is not fully understood yet. (2)

Distinct types of diabetes have been characterised (type 1, type 2, gestational diabetes mellitus and other types), from which type 1 and type 2 are the main categories. (2) Type 1 Diabetes (T1D) is caused by the autoimmune destruction of the β-cells of the pancreas, leading to no endogenous production of insulin. (3) Polydipsia, polyuria, sudden weight loss, extreme tiredness and diabetic ketoacidosis are some of the symptoms of T1D. (1, 2) Even though T1D can affect people of any age, it usually develops in the childhood and adolescence. (3, 4) Being less common than type 2 Diabetes (T2D), T1D affects 5 to 10% of all diabetics. (3) Moreover, *International Diabetes Federation* has estimated that approximately 542,000 children were living with T1D in 2015. Also, T1D was projected an increase around 3% every year in the number of people affected by type 1 DM. (1) T2D is characterized by insulin resistance, relative insulin deficiency and increased glucose production. (3, 5) It is highly influenced by the lifestyle so, physical inactivity and excess body weight are risk factors for the development of T2D. Also, ethnicity, family history of diabetes and advancing age are important risk factors to its development. (1, 3) Although it usually affects adults, the sedentary lifestyle adopted by children is leading to an increase of T2D incidence in younger people. (2) T2D present symptoms such as polydipsia, polyuria, sudden weight loss and blurred vision milder than T1D. (1) Due to the absence of severe symptoms in the beginning of the disease, T2D may take years to be recognised. In some cases complications associated with high blood glucose levels are already present when the person is diagnosed with T2D. (1, 2)

These complications can be observed in all types of diabetes as a result of consistently high blood glucose levels due to an incorrect control of blood glucose levels. Retinopathy, kidney failure, neuropathy and cardiovascular diseases, such as angina, stroke and heart attack, are some examples of important complications that can occur. (1, 3, 4, 6) Additionally, several studies have shown that the maintenance of normal blood glucose levels is of major importance for preventing and/or reducing the risk of future complications. (7-11)

In order to achieve an optimal control of glycaemia, patients must be submitted to the adequate therapy. It consists of insulin administration for T1D while hypoglycemic agents, such as sulfonylureas and metformin, are usually used for the treatment of T2D patients.

Additionally, in some cases insulin may also be required for treat T2D patients (either alone or in combination with hypoglycemic agents). (1, 4, 12, 13) Also, studies have demonstrated benefits in using transient intensive insulin therapy to rapidly obtain normoglycemia in some newly T2D diagnosed people before the use of standard therapy. (9) It should be noted that nonpharmacological interventions for T2D patients are also of major importance, such as the practice of physical exercise, adoption of a healthier diet and maintenance of a normal body weight. (1, 5) Also worthy of being mentioned is the growing evidence of improved glycemic control after bariatric surgery in T2D patients who also suffer from obesity. Consequently, several authors defend that this procedure has to be seen as an antidiabetic intervention. (14)

### 1.2. Insulin

Insulin is a peptide hormone produced by the β cells of islets of Langerhans in the pancreas. The human insulin molecule consists of two polypeptide chains, A and B with 21 and 30 amino acid residues respectively, linked by disulphide bridges (Figure 2). The insulin monomers aggregation into dimers and hexamers, due to the high concentration of zinc ions in pancreatic β cells, allows its efficient storage within vesicles. Having been released by exocytosis, hexamers dissociate in dimers and then monomers, which is the biological active form of insulin. (15-19)

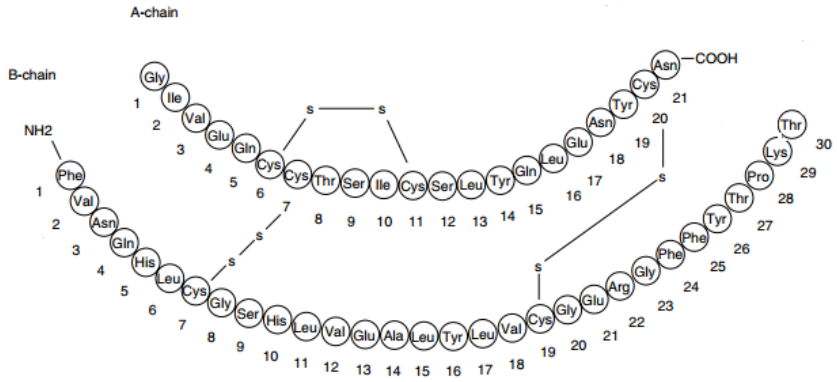


**Figure 2: Primary human insulin structure**. It consists of two polypeptide chains, A and B, linked by disulphide bridges. [Adapted from (19)]

In non-diabetic subjects insulin secretion by the β-cells of the pancreas occurs in response to various stimuli, such as hormones (glucagon, glucagon-like peptide-1), amino acids, fat-derived products and mainly glucose. (17, 20) Therefore, when blood glucose levels increase, the uptake of glucose through glucose transporter type 2 (GLUT2) by β-cells occurs.

As a result of glucose metabolism the adenosine triphosphate (ATP) levels rise. Increased production of ATP allows the closure of potassium channels and subsequently depolarization of the membrane, opening calcium channels and, thus influx of calcium. Increased calcium levels within the cell allows the exocytosis of insulin vesicles. (Figure 3) (17, 21)

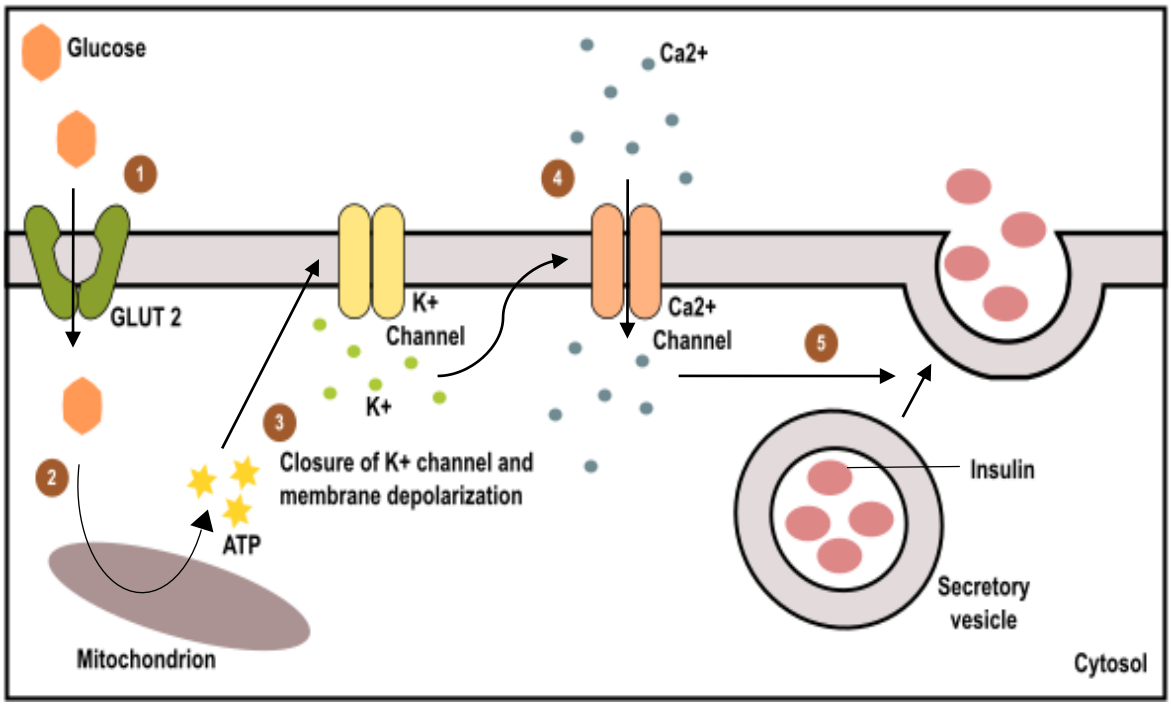


**Figure 3**: **Glucose-stimulated insulin secretion by the β-cells of the pancreas of a non-diabetic subject**. Glucose enters in the cell through GLUT2, being then metabolised. The subsequent increase of ATP levels causes the closure of $K^+$ channel and membrane depolarization, which results in the opening of $Ca^{2+}$ channels. The increased calcium levels, due to the influx through $Ca^{2+}$ channel, stimulates the exocytosis of insulin vesicles.

Once insulin binds to its receptor at the target cell (such as hepatocytes and adipocytes) the autophosphorylation of its receptor takes place. Therefore, the insulin signalling pathway is activated, leading to the recruitment and translocation of glucose transporter type 4 (GLUT4) to the plasma membrane. The increase in glucose transporters allows the efficient intake of glucose by the cell (Figure 4). (16) When blood glucose levels decrease insulin dissociates form its receptor, allowing the recycling of GLUT4 back into the cytosol. (16) This way insulin keeps glucose homeostasis by promoting the transport, intake, utilization and storage (synthesis of glycogen in the liver) of glucose by the cells. (16) Additionally, insulin inhibits proteolysis, autophagy and renal sodium excretion, stimulates the uptake of amino acids and potassium by the cells and regulate lipid metabolism. (16, 22)

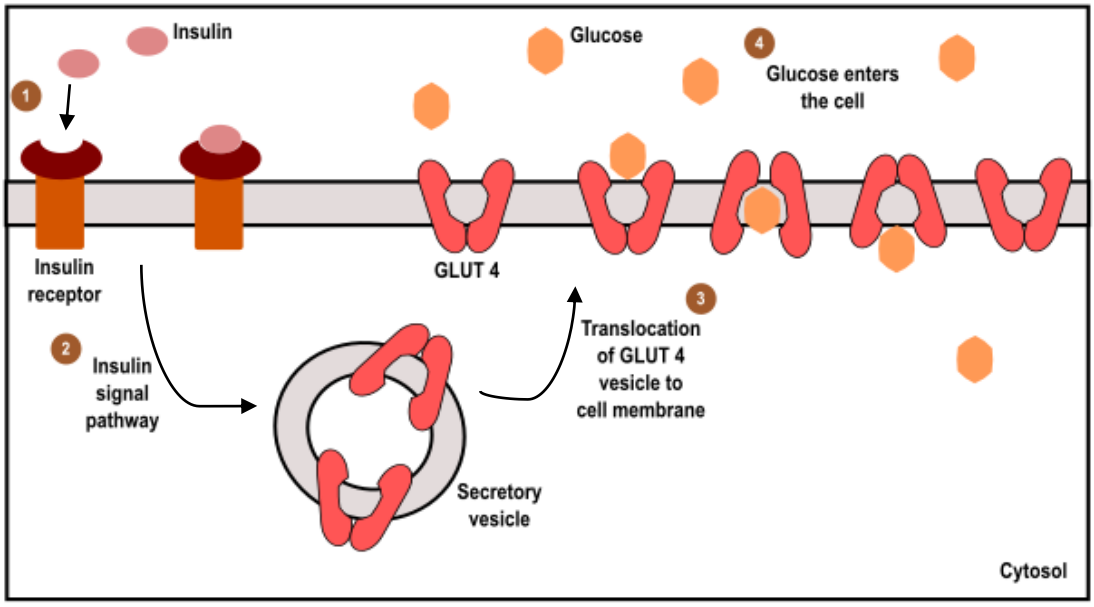


**Figure 4: Glucose uptake by a target cell (such as hepatocytes and adipocytes) of a non-diabetic subject.** The binding of insulin to its receptor causes the activation of insulin signal pathway. Consequently, GLUT4 is recruited to the plasma membrane facilitating the uptake of glucose.

Normal insulin secretion is characterized by basal insulin release throughout the day and prandial insulin release (release of insulin after carbohydrate ingestion). (22, 23) The basal insulin secretion occurs continuously, varies from 0.25 to 1.5U/h and is critical to control the endogenous glucose production by the liver as well as glycogenolysis and ketogenesis. (15, 20, 24) Due to the initial passage of insulin through the liver, about 50% to 60% of the insulin secreted into the portal system is removed, leading to a higher concentration of insulin in the portal vein than in a peripheral vein. (20) The prandial insulin release is responsible for the return of blood glucose levels to the basal levels after a meal. (15) It consists of a biphasic pattern with a fast release of insulin in a first phase (usually less than 10 minutes) and a second phase where a prolonged insulin release occurs as long as the hyperglycemia persists (usually 2-3 hours). (12, 20, 22)

### 1.3. Insulin Therapy

Since insulin was discovered scientists have tried to mimic the physiological profile of insulin action in healthy individuals through insulin administration in diabetic people. Nevertheless, because insulin is a peptide hormone, insulin therapy presents the same advantages and challenges as any other protein therapy. That is to say, although protein

therapy is usually highly efficient, due to the specific mechanism of action and high potency of proteins, dealing with their physicochemical and biological properties can be challenging. (25-27) Their high molecular weight and hydrophilic nature, instability due to their susceptibility to physical and chemical degradation, short *in vivo* half-life (mainly due to degradation by proteolytic enzymes and faster renal clearance) and possible immune responses may compromise the successful of the therapy. (18, 25, 27-30) Therefore, several attempts have been made to improve insulin therapy in order to mimic its physiological action, such as changes in insulin structure or formulations.

After several researchers tried to produce purified pancreatic extracts with hypoglycemic effect without success, Frederick Banting and Charles Best managed to prepare a successful pancreatic extract (nowadays known as insulin). They started their experiments in 1921 with a laboratory and dogs provided by J. Macleod. After J. B. Collip joined the group, they were able to produce a purified pancreatic extract. Their extract was first clinically used in 1922 at Toronto General Hospital, Canada, where a 14-year-old boy was successfully administered. (17, 31, 32)

Soon scientists begun to look for improvements in insulin therapy, namely the increase of the duration of action and the decrease of immunogenicity. The evolution of insulin therapy through the years is summarised in the Figure 5.

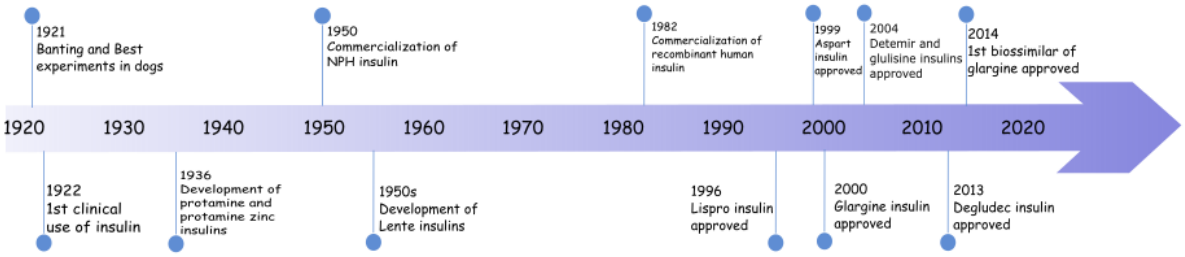


**Figure 5**: **Timeline for insulin developments**. (10, 23, 33, 34)

In the 1930s the prolongation of insulin action by its combination with protamine due to a reduction of insulin solubility was reported. Also, the addiction of zinc to insulin was found to allow a prolonged insulin effect. (18, 35) In 1950 the commercialization of Neutral Protamine Hagerdorn (NPH) insulin, produced by the addition of protamine to regular insulin at neutral pH, started. (13, 17) Additionally, in the 1950s, Lente insulins, with different ratios of amorphous and crystalline zinc insulin, were developed, allowing periods of action between 18 and 30 hours. (18, 35) In spite of the efforts made to improve the purification methods, most patients presented severe adverse reactions developing antibodies against the bovine or porcine insulins used. (23, 35) Not until the development and commercialization of

recombinant human insulin, did these reactions become more rarely. (18, 35) Since then animal insulins have been replaced by human recombinant insulin and different insulin formulations have become available to diabetic patients, allowing less severe adverse reactions and a more efficient control of blood glucose levels. (23)

The destabilization or stabilization of insulin hexamers by modifying the amino acid sequence of insulin, enabled the development of rapid and long acting analogues respectively. In other words, after a subcutaneous injection, the rate of dissociation of insulin hexamers, which accumulate in a subcutaneous depot, into dimers (and monomers) determines the onset and duration of action of insulin. (17, 36, 37)

To date, three rapid acting analogues have become available (lispro, aspart and glulisine). Different amino acid substitutions at insulin amino acid sequence (summarised at Table 1) have led to their faster onset and shorter duration of action. (15) Due to its rapid acting profile (Table 2), with an onset of action of less than 15 minutes, rapid acting analogues can be injected just before or after a meal. For that reason, a greater flexibility with mealtime for the patient is possible and so, insulin therapy became more comfortable and suitable for patients. (13, 15, 35)

Meanwhile, a prolonged action of insulin has been achieved by different approaches. The first long-acting insulin analogue to be approved was glargine in 2000 (Table 1). (34) As a result of the addition of two arginines to the C-terminus of the B-chain and the substitution of glycine for asparagine at position A21, the isoelectric point of insulin was increased from 5.4 in native insulin to 6.7 in glargine. (15, 38, 39) This alteration makes glargine less soluble at physiological pH thus, when injected this insulin analogue precipitates in a slow-dissolving depot of insulin hexamers, prolonging its absorption time and duration of action (Table 2). (15, 33, 36, 38, 39) Additionally, glargine hexamers are more stable and dense than regular insulin hexamers. (15) Another approach was used in the development of insulin detemir, whose long-acting profile results from the acylation of a lysine residue in position B29 with 14-carbon fatty acid (myristic acid) and removal of a threonine in position B30 (Table 1). (38, 40) Once injected, it binds to albumin at the site of subcutaneous injection, which is the main mechanism contributing for detemir long-acting profile (Table 2). Also, increased hexamer stabilization and hexamer-hexamer interaction prolongs detemir action. (36, 38, 41) Nevertheless, it is noteworthy that more patients need twice daily dosing with detemir than with glargine, which is usually administered once daily. (36, 42) The use of long-acting insulin analogues provides a basal insulin level that closely mimic the physiological levels, reducing the risk of hypoglycemic events observed with older insulin formulations. (17, 37, 38)

Interestingly, the patents for some of these insulin formulations, as well as its manufacturing process, have already expired or will expire in the next few years. Therefore, biosimilars (a biological product that is similar to an authorized biological reference product) are being developed and the first one, a biosimilar of glargine named Abasaglar (previously Abrasia), was approved by European Medicines Agency (EMA), in June 2014. Despite the new issues that biosimilars bring to discussion, such as potential immunogenicity, these new formulations represent new options for diabetic patients. (43, 44) Moreover, the presence of biosimilars of insulin analogues in the market may encourage the companies to invest in the research for new insulin formulations with better pharmacokinetic/pharmakodynamic profile. In fact, a second generation of insulin analogues is already being developed with an intense research in this area.

The first analogue of second generation being commercialized is Degludec, which was approved by EMA, in 2013 (Table 1). (34) In this analogue' structure $Thr_{B30}$ is absent and a thapsic acid was added to $Lys_{B29}$, via a gamma-glutamic acid spacer, in comparison to the structure of human insulin. (36, 42) It exhibits a flat and stable pharmacokinetic profile for more than 24 hours, allowing a glucose-lowering action for more than 42 hours (Table 2). Moreover, this new basal insulin analogue has been associated with lower hypoglycaemia risk and less weight gain than the first generation of basal insulin analogues. These characteristics are due to a multi-hexamer assembly after subcutaneous injection, leading to the formation of a subcutaneous depot that slowly release the active form of insulin. Also, the interaction of degludec with albumin in the bloodstream contributes to its long action profile. (36, 45, 46)

**Table 1: Current insulin analogues commercially available in Europe**. (34, 36, 37)

| Analogue | Brand Name | Company | Market approval | Modification in relation to human insulin structure | Category in relation to onset of action |
|---|---|---|---|---|---|
| Lispro | Humalog® | Eli Lilly | 30 April 1996 | $Pro_{B28} \rightarrow Lys$<br>$Lys_{B29} \rightarrow Pro$ | Rapid-acting |
| Aspart | NovoRapid® | Novo Nordisk | 7 September 1999 | $Pro_{B28} \rightarrow Asp$ | Rapid-acting |
| Glulisine | Apidra® | Sanofi-Aventis | 27 September 2004 | $Asn_{B3} \rightarrow Lys$<br>$Lys_{B29} \rightarrow Glu$ | Rapid-acting |
| Glargine | Lantus® | Sanofi-Aventis | 9 June 2000 | $Asp_{A21} \rightarrow Gly$<br>Addition of $Arg_{B31}$-$Arg_{B32}$ | Long-acting |
| Detemir | Levemir® | Novo Nordisk | 1 June 2004 | Absence of $Thr_{B30}$<br>Addition of myristic acid to $Lys_{B29}$ | Long-acting |
| Degludec | Tresiba® | Novo Nordisk | 21 January 2013 | Absence of $Thr_{B30}$<br>Addition of thapsic acid to $Lys_{B29}$ | Long-acting |

Abbreviations: Arg – Arginine; Asn – Asparagine; Asp – Aspartic Acid; Glu – Glutamic acid; Gly – Glycine; Lys – Lysine; Pro – Proline; Thr – Threonine

**Table 2: Insulin pharmacokinetic profiles**. (10)

| Insulin types | Onset of action | Peak of action | Duration of action |
|---|---|---|---|
| Regular | 30-60 min | 2-4 h | 5-8 h |
| NPH | 1-2 h | 4-12 h | 12-16 h |
| Lispro | 15-30 min | 30-90 min | 3-5 h |
| Aspart | 12-18 min | 30-90 min | 3-5 h |
| Glulisine | 12-30 min | 30-90 min | 3-5 h |
| Detemir | 1-2 h | 6-8 h | Up to 24 h |
| Gargine | 1-2 h | No pronounced peak | 20-26 h |
| Degludec | 30-90 min | No pronounced peak | >42 h |

Abbreviations: NPH – Neutral Protamine Hagerdorn

In summary, the typical insulin therapy for an insulin dependent patient consists of multiple insulin injections during the day, including injections of intermediate or long-acting insulin before bedtime, which provide a basal level of insulin, and fast-acting insulin at mealtimes, which control the higher levels of glucose after the meals. (47, 48) There are different options of insulin formulations with more or less prolonged duration of action available in the market in order to meet the needs of patients. Also worthy of mention is that pre-mixed insulin preparations, which combine long and fast-acting insulins in various proportions, are available in the market, allowing a more convenient regimen for diabetic patients. (13)

However, in spite of the improvements made in insulin therapy since it began, the ideal insulin formulation has not been achieved yet. The major disadvantages of current insulin therapy are the risk of hypoglycaemia and weight gain. (23) Additionally, poor patient compliance due to complex intensified regimens with multiple injections may also be an important issue. (15, 47) Therefore, explore novel approaches is essential in order to develop an insulin replacement regimen that precisely reproduce basal and prandial insulin secretion and so provide a higher patient compliance. (15)

# 2. NANOTECHNOLOGY AND HEALTH CARE

Nanotechnology (NT) has been defined as the ability to engineer and manufacture materials at the atomic and molecular level. It is noteworthy that although the *National Nanotechnology Initiative* has strictly defined NT on a scale of 1-100 nm size in at least one dimension, it often refers to structures bigger than several hundred nanometers. (49, 50)

As NT can be used to monitor, repair, construct and control human biological systems, its impact on medicinal areas is significant. (51) Medical diagnostics have become easier and faster due to the use of NT's techniques, such as nanobiosensors, molecular diagnostics and imaging by using nanoparticles based contrast materials. (52, 53) Not only medical diagnostics, but also therapy has been improved by NT. Tissue engineering, nanosurgery, nanorobotics and drug delivery systems are some examples of the use of NT in medicinal therapy. (51, 52) In particular, the use of NT for the development of drug delivery systems made possible the targeted delivery of drugs to specific cells or tissues, the co-delivery of two or more drugs and improved the delivery of large macromolecule drugs and poorly water-soluble drugs. (50, 54) Also worthy of mention is that NT is expected to produce new agents that combine diagnostic and therapeutic properties. That is to say, the same agent will be able to recognise, target and treat the biological error as well as provide real-time imaging for diagnostic. It should be noted that these properties are intrinsic in radioactive and magnetic nanoparticles so, they can easily be used for that purpose. (53) Moreover, it is believed that NT can decrease the cost of health care. (51)

## 2.1. Carrier-based Drug Delivery Systems (carrier-DDS)

The transport of drugs by carrier-DDS increases the half-live of the drug and decreases its toxicity. (26, 53, 55) Furthermore, the functionalization of the shell surface of the carrier allows the direct targeting to a specific group of cells, which is absolutely vital in the case of a localized disease, such as localized cancers. (26, 56) Another advantage of using these systems is the possibility of releasing the drug for a prolonged period of time. (57) To sum up, the controlled release of a drug by carrier-DDS, which is defined as the releasing of the drug at a controlled rate or to a specific location for an extended period of time (57, 58), allows a reduced dosing frequency, decreased side effects and increased efficacy and consequently improves patient compliance. (26, 30, 58, 59)

A doxorubicin liposome, Doxil®, was one of the first approved drugs based on a carrier-DDS for the treatment of cancers, such as refractory ovarian carcinoma and Kaposi sarcoma. (50, 53) In fact, there are intense research with carrier-DDS for the treatment of cancers. (53) In addition to oncology, autoimmune diseases, mental disorders and metabolic diseases, among others may benefit from using these systems. (29, 49) Therefore, researches have been developed and not only chemical drugs, but also therapeutic peptides/proteins have been delivered by carrier-DDS. (60) The use of carrier-DDS is especially important when delivering peptide/protein drugs because dealing with their physicochemical and biological properties is sometimes challenging as aforementioned. This strategy of peptide/protein delivery protect them from degradation as well as increases their stability. (55, 60) Also worthy of mention is that other approaches have been used to surpass protein limitations. For example, chemical modification of macromolecules by aminoacid substitution, as performed in some insulin analogues. Also, attachment of compounds such as polyethylene glycol (PEG) and fatty acids has been used. (25, 30, 61) Nevertheless, the use of carrier-DDS is advantageous because it avoids any chemical or fusion modification of the peptide/protein. (59) Therefore, using carrier-DDS is seen as the most promising approach to peptide/protein delivery. (62)

For diabetic patients NT represents a promise of an easier live in the future. Not only has NT been investigated for the development of less invasive methods of glucose monitoring, but also it has been explored for the treatment of DM related complications, such as diabetic retinopathy. (49, 51) Moreover, insulin delivery systems based on micro- and nanoparticles have been studied in order to achieve a controlled delivery of insulin, reducing the frequency of insulin injections and subsequently increasing patients' compliance. Also, it is expected that the better control of glucose levels by using insulin delivery systems will decrease the incidence of DM related complications. (51, 55, 63, 64)

Several types of carriers have been developed and it is now well established that an ideal carrier should fulfil a set of requirements. Briefly, it should be made of a non-cytotoxic, biocompatible and biodegradable material through a non-complex preparation method that does not affect protein bioactivity. Besides, the chemical and physical protein's degradation must be avoided by the carrier. Moreover, it should be easily modified, using conventional chemical techniques, allowing its targeting to the target area as well as preventing an non desired immunological response. Not only should carriers' surface modification (i.e. nature and density of the ligands) be considered but also its size, charge and surface hydrophilicity must be measured. Also, ideally, the carrier should present a high loading efficiency and be

able to release the protein in a sustained manner. Last but not least, the carrier should be easily manufactured in large quantities in order to be clinically used. (27, 30, 50, 62, 65)

Carriers are considerably diversified in relation to their chemical compositions and physicochemical properties. There are, for example, polymeric, lipid-based, metallic and ceramic carriers, among others. (49, 66) Due to carrier's diversity, they present different limitations and advantages (Table 3), which makes them more or less suited for delivery of different drugs or through various delivery routes. (67) Apart from their different composition, there are carriers with various morphologies: micelles, vesicles, particles, tubes and gels. Also, it should be mentioned that these systems have been characterized in sizes ranging from nanometre to micrometre. (62) Importantly, it has been reported that both morphology and size of a carrier may influence his behaviour after administration. For example, nanoparticles not bigger than 50 nm present a more efficient cellular uptake and elongated nano-carriers have slower clearance. (68) In addition to carrier's size and shape, carrier's surface chemistry is another determining factor for its biodistribution pattern, cellular uptake and clearance mechanisms. (69) Therefore, there are several approaches that can be used to improve drug delivery by means of carriers and the choice of the most appropriate depends on the physicochemical properties of the peptide/protein, the desired route of administration and some clinical aspects of the therapy. (70)

**Table 3: Some types of carriers.**

| Carrier | Characteristics | Advantages | Limitations |
|---|---|---|---|
| **Liposomes (62, 68, 71-73)** | Comprised of one or more phospholipid bilayers surrounding internal aqueous compartments. | Biocompatible, low toxicity, ease of surface chemistry modification, ability to deliver both hydrophilic and hydrophobic drugs. | Low protein loading efficiency, difficulty of scale up, *in vivo* instability of the formulation due to interaction with high-density lipoproteins in the blood, capture by the reticuloendothelial system. |
| **Solid lipid nanoparticles (54, 71-75)** | A solid lipid matrix with a hydrophobic core where the drug is enclosed either dissolved or dispersed. | Biocompatible, biodegradable, high drug loading efficiency, improved protein stability on storage and release, ability to carry both lipophilic and hydrophilic drugs, easy production and scale-up. | Undesired particle agglomeration or coagulation resulting in "burst release". |
| **Polymeric particles (26, 60, 62, 65, 71, 76)** | Solid particles composed of natural, semi-synthetic or synthetic polymers where the drug can be uniformly dispersed (spheres) or enclosed inside the core (capsule). Depending on the size, they can be classified as nano or microparticles. | Biocompatible, biodegradable, several techniques are available to produce polymeric particles, possibility of properties optimization by using several polymeric materials and targeting ligands. | Protein instability during formulation process, possible particle aggregation in handling, storage and administration. |
| **Polymeric micelles (62, 68, 72, 76-78)** | Produced by self-assembly of amphiphilic polymers resulting in a hydrophobic core surrounded by a hydrophilic surface. | Biocompatible, low toxicity, high drug loading efficiency, high stability, optimization of loading and release profiles by using several polymeric materials. | The loaded drug may be released by diffusion before reaching the target site. |

Despite the improvement of carriers' characteristics, some challenges still remain and different approaches are being tested and used in order to overcome as much problems as possible. For instance, rapid clearance of the carriers from the blood by the mononuclear phagocytic system (MPS) may sometimes occur. (29, 49, 66, 79) Because of it there are more and more interest in the development of carriers able to avoid the recognition by the MPS, for example the PEGylation of the carrier is used with this purpose. (66)

Another major concern is the possible instability of the encapsulated peptide/protein resulting in its denaturation, aggregation or deamination which may cause therapeutic inactivity and unexpected side effects. This instability may be due to the intrinsic characteristics of the protein, the nanomaterial that constitute the carrier (for example, polymer-peptide/protein interaction may be one of the causes) or the formulation method used. (60, 66, 80-82) Several factors during the formulation process may have an impact on peptide/protein stability, such as temperature, pH, shaking, water-oil interface, peptide/protein concentration or the presence of strong solvents or contaminants. (60, 61, 70) These factors differ from one preparation method to another. (60) Therefore, it is essential to choose not only the most appropriate nanomaterial, but also the most suited preparation method. Also, the improvement of formulation procedures is vital in order to solve this problem. It should be noted that the stabilization of the peptide/protein during the formulation process have been tried by different approaches, such as the addition of stabilizers or modifications of the peptide/protein structure, among others. (29, 60, 66, 82) It is worth mentioning that the maintenance of protein stability is a concern not only in the formulation process, but also during storage and release from the carrier. (71, 83)

In addition to the improvement of preparation methods as aforementioned, the development of better techniques to evaluate the stability of the peptide/protein in the carrier is essential. (71) Techniques such as high performance liquid chromatography, size exclusion chromatography, high performance liquid chromatography-mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, capillary electrophoresis and western blot have been used to evaluate protein stability. Particularly, Fourier transform infrared spectroscopy has become a popular, non-invasive method used to analyse the structure of encapsulated peptides/proteins. (60, 82, 84) Even though, several techniques have been used to assess protein stability, every time the extraction of the protein is necessary prior to the analysis, the results might be influenced by it. For example, the extraction media is sometimes responsible for irreversible conformational changes in the peptide/protein or the dissolution or formation of

peptide/protein aggregates. (84) Consequently, it is vital to perform the evaluation in conditions that do not alter the protein. (82) It should also be mentioned that the protein stability is evaluated by its biological activity or immunogenicity when the evaluation is performed *in vivo*. (60, 82)

Toxicity concerns about the usage of carriers to deliver peptides/proteins have also been raised. (68) The carrier's size might have influence on its toxicity. For instance, nanoparticles have been reported to present higher toxicity than microparticles (MPs) because of a more favourable cellular uptake of the nanoparticles. (54) It was suggested the induction of reactive oxygen species as their toxicity mechanism. (85) Moreover, the material that comprises the carrier or the products of its degradation may present toxicity. (53) In addition, the incomplete removal of potentially toxic solvents used in the preparation procedure may also result in toxicity problems. (81)

Also worthy of mention is that the correlation between the *in vitro* and *in vivo* studies' results is not always observed. Accordingly, new developments in this area should be performed. (27, 29, 71)

### 2.1.1. Polymer-based Carriers

Even though more and more new types of carriers are being developed, lipid and polymer based carriers as well as polymer-lipid conjugates have been the most widely used as a drug delivery system. (50, 68) Hereafter, only polymeric carriers will be considered since this thesis intend to analyse the use of polymeric carriers to deliver insulin through the parenteral route.

On the one hand, these carriers offer several advantages (Table 3), such as an increased stability. In fact, in comparison with lipid-based carriers, polymeric carriers are usually more suited for drug delivery in a large variety of conditions. (62, 65) Besides, the possibility of modification of their physicochemical properties, biological behaviour and drug release properties by using different polymers and targeting ligands, makes them versatile carriers. (60, 62, 65) On the other hand, some difficulties regarding handling, storage and administration due to possible aggregation as well as protein instability during the formulation procedure may limit their usage. (26, 60, 62)

There are polymer-based carriers with different morphologies, sizes and composition. Particularly, polymeric MPs and nanoparticles (NPs) have been studied for the delivery of peptides/proteins. Polymeric MPs range in sizes from 1 to 250 μm whereas polymeric NPs vary in size from 10 to 1000 nm. (49, 65, 86) It is worth mentioning that polymeric NPs' smaller size

may represent an advantage in relation to polymeric MPs since, owing to polymeric MPs larger size, polymeric MPs are most likely to suffer opsonisation than polymeric NPs. Therefore, a longer time in circulation is observed for polymeric NPs. Also, polymeric NPs present a considerably higher cell uptake than polymeric MPs. (27, 62) Thus, the particle's size affects their biodistribution and clearance. (68) Besides, in practical terms, it may also be required the use of larger gauge needles for the administration of polymeric MPs. (59) Interestingly, polymeric MPs have been more studied than polymeric NPs for peptide/protein delivery and, so, it should be emphasised that it is often more challenging to achieve a sustained release from polymeric NPs than from polymeric MPs. It may be due to a less efficient drug loading and/or a faster drug release observed for polymeric NPs. (74, 86) Both polymeric MPs and NPs can be of two types: spheres or capsules (Figure 6). In micro or nanospheres, the drug and the polymer are uniformly dispersed while in micro or nanocapsules, the drug is enclosed inside the polymer cavity. (49, 60, 65, 72, 76) It should be noted that both polymeric MPs and NPs have been explored for parenteral administration. However, polymeric NPs have mostly been administered by intravenous route, whereas polymeric MPs have mainly been administered by intramuscular or subcutaneous routes. (62, 86, 87)

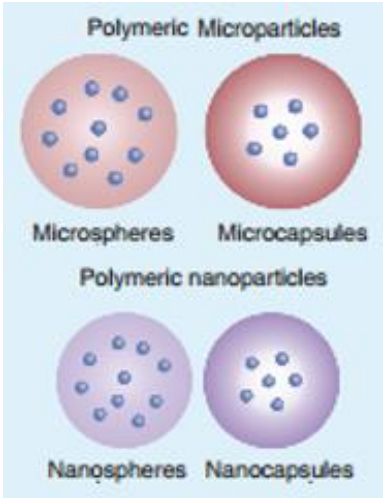


**Figure 6: Polymeric micro and nanoparticles**. [Adapted from (71)]

### 2.1.1.1 Types of polymers

Polymeric MPs and NPs have been made up with a large variety of polymers through several formulation methods. (27) Ideally, the polymers used as a peptide/protein delivery system should present biocompatibility, non-toxicity, biodegradability and controlled release potential.

Moreover, the polymer must neither affect the pharmacological properties of the encapsulated molecule, nor present adverse side effects (either the polymer or its degradation products). (58, 60, 88) So, in addition to the controlled release potential, the polymer must be compatible with the encapsulated peptide/protein and the body. (27)

Biodegradable polymers are advantageous due to their *in vivo* enzymatic or non-enzymatic degradation into biocompatible and non-toxic products (such as non-toxic alcohols and acids) which are eliminated from the body by normal metabolic pathways. (29, 30, 56) Therefore, the use of biodegradable polymers improves tissue compatibility and reduces cytotoxicity. (72) Moreover, the release of the encapsulated protein is mostly dependent on the degradation kinetics of the used polymer. (89) It should be noted that non-biodegradable polymers with good biocompatibility have also been used for drug delivery. (83, 90)

Both natural and synthetic polymers have been used to formulate polymeric particles (Table 4). (65) Some widely employed natural polymers for peptide/protein delivery are the polysaccharides starch and alginate and the protein collagen. (71) Despite natural polymers started as the main focus of research as biodegradable systems, attentions have started to focus on synthetic biodegradable polymers. (49) It was observed that, in contrast to natural polymers, synthetic polymers allow a controlled drug release over periods of days to weeks. (65, 66) At the present, the more frequently used synthetic polymers for peptide/protein delivery are biodegradable polyesters (such as poly(lactic acid), PLA, poly(lactic-co-glycolic), PLGA, poly-ε-capralactone, PCL) and poly(ortho esters). (71, 85, 87) From these polymers, PLGA and PLA has been the most popular, being used in an enormous number of studies due to its highly biocompatibility and biodegradability as well as their use in medical devices (such as bioabsorbable sutures and bone plates) for a long time. (30, 67, 71, 83-85)

Considering the diversity of polymers available, the choice of the most suited polymer for the delivery of a specific peptide/protein is determined by the peptide/protein physicochemical properties, polymer properties (namely, toxicity) and the intended clinical usage. (89)

**Table 4: Examples of natural and synthetic polymers used as delivery systems**.

(30, 58, 60, 66, 70, 80, 83)

| Classification | Polymer | |
|---|---|---|
| **Natural polymers** | Protein-based polymers | Collagen<br>Albumin<br>Gelatin |
| | Polysaccharides | Starch<br>Alginate<br>Chitin/Chitosan<br>Hyaluronic acid and derivatives<br>Dextran<br>Ciclodextrins<br>Cellulose |
| **Synthetic polymers** | Polyesters | Polylactides<br>Polyglycolides<br>Poly(lactide-co-glycolides)<br>Polycaprolactones |
| | Polyanhydrides | Poly(fumaric-co-sebacic) anhydride |
| | Others | Polyorthoesters<br>Polyphosphazenes<br>Pseudopolyamino acids<br>Polycyanoacrylates Poly(methyl methacrylates) |

### 2.1.1.2. Formulation methods

Ideally, the formulation method should be able to produce a high peptide/protein encapsulation efficiency as well as a low burst release. It is also desired a high yield of particles of the wanted size that do not aggregate. Besides, it should maintain the bioactivity of the peptide/protein, avoiding harmful solvents or other severe formulation conditions (such as high temperatures). A simple, reproducible and scalable method would also be preferred. (29, 82) As previously mentioned, several preparation methods have been employed in the formulation of polymeric particles. (27, 70) Each of them present several advantages and limitations. (89)

Therefore, in order to choose the most appropriate method for each situation, the solubility and stability of the peptide/protein in different solvents as well as the stability of the final product must be considered. Moreover, other factors influence the selection of the formulation method, such as the desired particle size and the reproducibility of the release kinetic profile. (62, 89, 91)

Single emulsion, spray drying, coacervation and nanoprecipitatation, among others, are some examples of methods widely used for peptide/protein encapsulation in polymeric particles. (25, 29, 62, 66, 89) However, multiple emulsion solvent evaporation methods have been the most employed for protein encapsulation in polymeric MPs and NPs. (65, 80, 82, 86) The most commonly used is water-in-oil-in-water (w/o/w) double emulsion. In this method, the aqueous solution of the protein is firstly dissolved in an organic solution of the polymer while shearing forces are used (ultrasonication/homogenization), creating the primary emulsion. It should be noted that ethyl acetate and methylene chloride are the most frequently organic solvents used. Secondly, the dispersion of the primary emulsion in an aqueous phase, which contains an appropriate stabilizer such as polyvinyl alcohol, forms a w/o/w double emulsion. Finally, the removal of the organic solvent is performed by evaporation, for example through rotary evaporator. It is noteworthy that solvent extraction may also be used for removal of the organic solvent. (25, 29, 82, 89) In spite of being the most popular protein encapsulation method for polymeric particles, it presents some limitations. The main issue is the peptide/protein instability due to its exposure to the aqueous organic interface and shearing forces used. It is now well established that peptides/proteins are likely to migrate and adsorb to the water-oil interface, exposing their hydrophobic area, which is usually hidden in their active state, to the organic solvent. As a result, denaturation of the encapsulated peptide/protein may happen and, consequently, its bioactivity is lost and immunogenic reactions as well as toxicity may occur. (60, 65, 77, 89)

It is noteworthy that not only double emulsion solvent evaporation method, but also the other methods of nano or micro encapsulation may induce protein instability due to mechanical, thermal or chemical stress. (86) Therefore, being peptide/protein instability a problem to several formulation processes, various approaches have been used in order to improve peptide/protein stability.

Sometimes variations to a main method have been used, for example solid-in-oil-in-water (s/o/w) emulsion is sometimes used as an alternative to water-in-oil-in-water double emulsion, because in s/o/w a solid-state of the protein is used. Since the presence of water-oil interface is

avoided and the solid-state protein maintain its activity in the organic solution, s/o/w allows overcoming w/o/w major limitation. It should be noted that oil/oil emulsion have also been developed. (60, 77, 82) Also, modifications to the polymer or the protein have been employed. For example, PLGA and PLA modification with hydrophilic or hydrophobic segments may improve peptide/protein stability during formulation process. Besides, proteins may be PEGylated, preventing their denaturation by reducing their tendency to adsorb to the water-oil interface. (60, 82) Another approach is the reduction of the exposure time to shearing or its intensity. If possible this approach is combined with the use of additives. (82) In fact, the addition of stabilizers, such as surfactants, PEG, sugars, proteins, etc., during the formulation process is the most common strategy. (60, 65, 66, 82, 86)

As previously discussed, both the type of polymer that compose the particle and the formulation method may influence significantly the particles' properties, such as the rate and duration of peptide/protein release. (65, 71) Therefore, a judicious choice of the polymer as well as the formulation method is essential.

#### 2.1.1.3. Commercialized encapsulated peptides/proteins

Several peptide/protein formulations for controlled release making use of polymeric carriers have been approved for clinical use (Table 5). For example, Nutropin Depot® from Genentech, a PLGA microsphere formulation of recombinant human growth hormone, received market approval in 1999. It was indicated for the treatment of recombinant human growth hormone deficiency and a single subcutaneous injection was able to achieve a sustained-release over one month. However, its sale was discontinued in 2004 because of the manufacturing and commercialization costs. (29, 30, 67, 92) Some other examples of parenteral sustained release peptide/protein formulations are Lupron Depot® (leuprolide acetate loaded PLA microspheres) and Trelstar™ Depot (triptorelin pamoate loaded PLGA microspheres), used in the treatment of prostate cancer, among others. (25, 58, 83, 86)

**Table 5: Some examples of approved peptides/proteins delivered by polymeric carriers**. (25, 58, 83, 86)

| Trade Name | Peptide/Protein | Carrier | Indication | Company |
|---|---|---|---|---|
| **Lupron depot®** | Leuprolide acetate | PLA MP | Prostate cancer | Takeda-Abott |
| **Nutropin depot®** | Recombinant human growth hormone | PLGA MP | Growth hormone deficiency | Genentech |
| **Sandostatin LAR® depot** | Octreotide acetate | PLGA MP | Acromegaly, GH suppression anticancer | Novartis |
| **Somatuline® LA** | Lanreotide | PLGA MP | Acromegaly | Ipsen |
| **Trelstar™ depot** | Triptorelin pamoate | PLGA MP | Prostate cancer | Pfizer |
| **Suprecur® MP** | Buserelin acetate | PLGA MP | Prostate cancer | Aventis |

**Abbreviations:** MP – Microparticle; GH – Growth hormone; PLA – Poly(lactide); PLGA – Poly(lactide-co-glicolide)

# 3. PARENTERAL ADMINISTRATION OF ENCAPSULATED INSULIN

Nowadays most biopharmaceuticals are administered by parenteral routes, which include but are not limited to subcutaneous (SC), intramuscular (IM) and intravenous (IV) injections. From these, SC injections are the most commonly used, due to their suitability for easy self-administration, while IM and IV routes are mostly used for vaccines and monoclonal antibodies administration, respectively. (30, 70, 71, 93) Regardless of the parenteral route chosen, most peptide/protein drugs present a short *in vivo* half-life. Specifically, insulin, which is usually administered by SC injection, exhibit a half-life of 4-5 minutes after parenteral administration. (48, 63, 93) Therefore, multiple daily injections are required for the treatment of DM, as for other chronic diseases, in order to achieve effective therapeutic levels. (60, 94)

However, repeated SC injections are often associated with pain, tenderness, local tissue necrosis, microbial contamination and nerve damage, resulting in poor patient compliance. (16, 63, 71, 80) It is worthy of mention that although pain is still associated with injections, it has been considerably reduced by technological advances such as insulin pens. Besides, in a study which objective was to assess the pain associated with injection of insulin, it was concluded that the median pain score of these injections was less than 10% of maximum pain, meaning insulin injection is relatively painless. Despite these findings, there is no doubts that some people has a psychological aversion to injections, compromising patient compliance. (13, 18, 93) In addition to the fear of injections, patients also feel worried about hypoglycemic episodes, weight gain and accommodation of the scheduled insulin doses in their daily routine. (23, 40, 95)

The lack of patient compliance represents an important factor that leads to poor glycemic control that may result in DM-related complications, as already discussed. (47) Therefore, it is vital to improve patient compliance. Parenteral delivery of encapsulated insulin may help to solve this problem by prolonging insulin delivery for weeks or months. Consequently, the frequency of insulin injections may be reduced, increasing glycemic control by increasing patient compliance. (63, 96)

Furthermore, the prolonged action of insulin by encapsulation would be favourable for the maintenance of the insulin concentration within the therapeutic window when compared to the current therapy. That is to say, through multiple daily SC injections of insulin the plasma insulin concentration may achieve levels lower or higher than the therapeutic levels (Figure 7). (97)

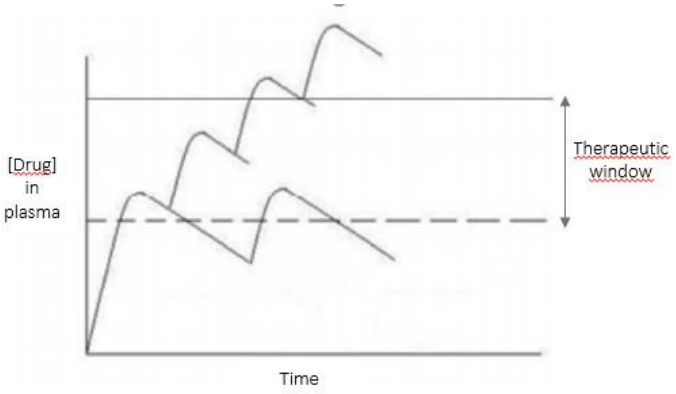


**Figure 7: Drug concentrations in plasma after multiple administration in a chronic disease**. [Adapted from (97)]

In contrast, an ideal prolonged delivery of insulin would be able to initially release a sufficient amount of insulin to achieve a therapeutic effect and then extend this therapeutic activity (Figure 8). (97)

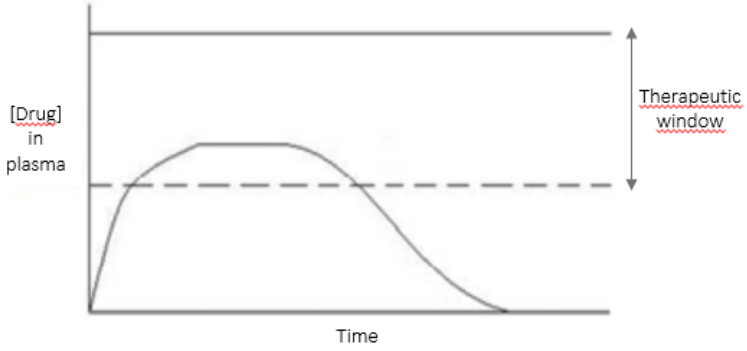


**Figure 8: Drug concentrations in plasma after administration of a prolonged release formulation**. [Adapted from (97)]

In summary, the encapsulation of insulin may extend its *in vivo* half-life by prolonging its delivery. Therefore, this formulation may not only decrease the frequency of injections but also, increase the efficacy of the therapy, resulting in increased patient compliance. (71) Table 6 summarizes several studies performed with polymeric MPs or NPs with potential antihyperglycemic effect.

**Table 6: Physicochemical characterization of insulin-loaded polymeric particles with potential antihyperglycemic activity after parenteral administration**.

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Anti-hyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **Poly-phosphazene MP** | Suspension-solvent evaporation (A)<br><br>Double emulsion-solvent evaporation (B)<br><br>Suspension/double emulsion-solvent evaporation (C) | 55000 (A)<br><br>43000 (B)<br><br>43000 (C) | - | STZ-induced diabetic SD rats | SC | *In vitro:*<br>A and C:<br>-Lower initial burst release<br>-Lower amount of insulin released | A and C:<br>-Peak: 2h<br>-Initial BGL after 300-400h<br><br>B:<br>-BGL decreased 80% after 1h<br>-Initial BGL after 1000h | Method B showed the best antihyperglycemic activity. | (98) |
| **PEG/PLA NP** | Gas antisolvent $CO_2$ precipitation | 400-600 | - | Balb/c mice | SC | *In vitro:*<br><3% was released in 80h.<br><br>Low MW PEG allowed a slow but constant release of insulin over 1500h. | Insulin maintained >80% of its bioactivity. | The bioactivity of insulin was maintained.<br><br>Low efficiency of insulin release. | (99) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PMAA/PLGA MP**<br><br>**PLGA MP** | Modified w/o/w emulsion solvent evaporation | PMAA/PLGA MP: 89000<br><br>PLGA MP: 64000 | PMAA/PLGA MP: 68<br><br>PLGA MP: 39 | STZ-induced diabetic SD rats | IP | *In vitro:*<br>PMAA/PLGA MP:<br>- 26% of the insulin was released in 24h<br><br>PLGA MP:<br>- 18% of the insulin was released in 24h<br><br>Both MP:<br>- a second phase of slow release phase was observed | PMAA/PLGA MP showed bioactivity, reducing BGL for 4 days. | PMAA/PLGA MP:<br>- Higher efficiency of encapsulation<br>- Reduced BGL for 4 days<br>- Higher initial burst release when comparing with PLGA MP. | (100) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PLGA MP** | Modified o/w single emulsion solvent extraction/ evaporation process | 65000 | 85-100 | STZ-induced diabetic SD rats | SC | *In vitro:* A 4-day lag period was observed after an initial release of <0.5% in the first day.<br><br>93% was released over 16 days.<br><br>*In vivo:* Initial burst release similar to that observed *in vitro*.<br><br>Able to maintain steady state serum insulin levels in the 50-75µIU/mL range in a multiple dose study, | BGL were maintained below 200mg/dL from day 3 to day 10 after a single dose administration. | Initial burst release of <1% in the first day.<br><br>Serum insulin levels were maintained in the 50-75 µIU/mL range, in a multiple dose study (doses given at 7-day intervals), | (101) |
| **PLA MP**<br><br>**PLGA MP** | Double emulsion solvent evaporation | PLA MP: 41000<br><br>PLGA MP: 53000 | PLA MP: 75.2<br><br>PLGA MP: 79.6 | - | - | *In vitro:* High initial burst release observed for both PLA MP (66%) and PLGA MP (71%), during the first 6h. | - | Structural alterations in insulin during the erosion of both polymers. | (102) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **p(FASA) MP** | Phase inversion micro- and nanoencapsulation | 5900 | - | Diabetes-Prone BB rats | SC | *In vitro:* 35% released in the first 24h.<br><br>40% had been released after 12 days. | Observed for 3 different doses of insulin (44.8U/Kg, 53.7U/Kg and 66.5U/Kg). | Able to prolong the duration of insulin action. | (103) |
| **PLGA MP** | Double emulsion solvent evaporation | 24000 | >90 | Diabetes-Prone BB rats | SC | - | Gradual reduction of BGL after the first injection, followed by an increase in BGL resulting in hyperglicemic levels before the second injection.<br><br>200U/Kg:<br>- transient hypoglycemia<br><br>100U/Kg: insufficient hypoglycemic effect<br><br>125U/Kg:<br>- best antihyperglycemic results. | Administration every 2 weeks was able to keep rats alive without additional treatment. | (104) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PCL MP**<br><br>**PCL NP** | Double emulsion solvent evaporation | PCL MP: 28000-43000<br><br>PCL NP: 750 | PCL MP: 31.8-53<br><br>PCL NP: 28.3 | - | - | *In vitro:*<br>PCL MP:<br>- 45-60% released after 12h<br><br>PCL NP:<br>- <40% released after 12h | - | PCL NP released less insulin than PCL MP in 12h. | (105) |
| **PLGA NP** | Double emulsion solvent evaporation | Stabilized PLGA (50:50) NP: 1146<br><br>Stabilized PLGA (85:15) NP: 502 | Stabilized PLGA (50:50) NP: 24.8<br><br>Stabilized PLGA (85:15) NP: 24.5 | STZ-induced diabetic Wistar rats | SC | *In vitro:*<br>Higher amount released when the stabilizers, sodium bicarbonate, trehalose and Pluronic® F68, were used. | Stabilized PLGA (85:15) NP:<br>- Exhibited a more sustained antihyperglycemic effect over 72h when compared to stabilized PLGA (50:50) NP. | Stabilizers:<br>- Increased mean particle size<br>- Decreased efficiency of encapsulation<br>- Increased insulin release rates<br>- Prolonged antihyperglycemic effect | (106) |
| **HGC NP** | Dialysis | 184 | 83.0 | STZ-induced diabetic SD rats | SC | *In vitro:*<br>Initial burst release of 40% within 12 hours, followed by a slow release.<br><br>Over 60% released after 120h. | HGC NP:<br>- Lower initial reduction of BGL when compared with regular insulin<br><br>- Antihyperglycemic activity for up to 24h<br><br>- normal BGL were achieved after oral glucose load (24h post administration). | Prolonged antihyperglycemic effect when compared with regular insulin. | (107) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PLGA NP** | Modified spontaneous emulsion solvent diffusion | 230-250 | - | ddY mice | SC | *In vitro:*<br><br>In deionized water medium:<br>- Initial burst release <15% for 0.5h<br>- Incomplete release (<30%) over 6h.<br><br>In PBS medium:<br>- Initial burst release of 60-65% for 0.5h.<br>- >85% released over 6h. | Higher bioactivity than free insulin. | The use of SDS-insulin reduced the mean particle size when comparing with insulin.<br><br>Significant differences observed for in vitro insulin release in deionized water and PBS medium. | (108) |
| **PLGA MP** | Atomization through a monoaxial nozzle ultrasonic atomizer | 50000 | 49.0 | STZ-induced diabetic SD rats | SC | *In vitro:*<br>Initial burst release of 20% within 24h.<br><br>About 50% released within 30 days.<br><br>*In vivo:*<br>Initial burst release similar to that observed *in vitro.*<br><br>Sustained release for 30 days. | PLGA MP:<br>- Normal BGL 3h after administration.<br><br>- Able to maintain BGL at 100-200 mg/dL for 55 days.<br><br>Free insulin:<br>- Hyperglycemic values after 3 days. | Able to achieve and maintain normoglycemic levels in contrast with insulin solution. | (63) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PLGA MP** | s/o/o emulsion | 1000-10000 | NP insulin-loaded PLGA MP:<br>-PLGA 10k: 99.4<br>-PLGA 25k: 90.5<br>-PLGA 90k: 100.8<br><br>Commercial insulin-loaded PLGA (10k) MP: 50.1 | AM-induced diabetic Wistar rats | SC | *In vitro:*<br><br>NP insulin-loaded PLGA MP:<br>-Decreased initial burst release (PLGA 10k: 7%; PLGA 25k: 6%; PLGA 90k: 5%)<br>-Prolonged release for more than 60 days.<br><br>Commercial insulin-loaded PLGA (10k) MP:<br>-Initial burst release of 44%.<br>-Fast release that lasted for 10 days. | Insulin maintained its bioactivity. | NP insulin-loaded PLGA MP:<br>- Increased encapsulation efficiency.<br>- Reduced initial burst release.<br><br>Small influence of the MW of PLGA in encapsulation efficiency and *in vitro* insulin release. | (109) |
| **PLGA MP**<br><br>**PLA MP** | Multiple emulsion-solvent evaporation | 37000 | PLGA MP: 71.5<br><br>PLA MP: 65.0 | AM-induced diabetic Wistar rats | IM | *In vitro:*<br>PLGA MP:<br>-13.96% released in the first 30 minutes.<br>-75.35% released after 168h.<br><br>PLA MP:<br>-13.38% released in the first 30 minutes.<br>-67.54% released after 168h. | PLGA MP:<br>- 53.86% reduction in BGL.<br><br>PLA MP:<br>- ~40% reduction in BGL.<br><br>Both MP:<br>- Decreased BGL for more than 168h. | Reduction of BGL with both MPs.<br><br>Faster insulin release for PLGA MP. | (110) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PLA/PEG MP** | Double emulsion solvent evaporation | 3070 | 42.7 | STZ-induced diabetic SD rats | SC | *In vitro:* Initial burst release of 18.79%.<br><br>Constant release over 1 week.<br><br>*In vivo:* Constant release for 1 week after a single SC injection. | - | Optimized formulation able to reduce the initial burst release to 18.79%.<br><br>Potential as a controlled release formulation (*in vivo* studies). | (64) |
| **Heparin coated PCL-PMA NP** | Double emulsion solvent evaporation | 357 | 83.9 | STZ-induced diabetic Wistar rats | IV | *In vitro:* Initial burst release of 62% within 5 minutes.<br><br>Constant release for 24h.<br><br>In 24h, 70% was released. | - | Increased half-life of up to 116h.<br><br>High initial burst release. | (79) |
| **p(CPP:SA) MP** | Double emulsion solvent evaporation | 49800 | 84.8 | STZ-induced diabetic SD rats | SC | *In vitro:* Initial burst release of 22%.<br><br>Prolonged slower release for over 1 month.<br><br>*In vivo:* Insulin release for 35 days. | BGL below 300 mg/dL for 7 days.<br><br>Pretreatment BGL at day 35. | Increased efficiency of encapsulation.<br><br>Reduced initial burst release.<br><br>Antihyperglycemic activity *in vivo*. | (48) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PLGA MP** | Double emulsion | 14600 | 94.0 | STZ-induced diabetic Sprague Dawley rats | SC | *In vitro:*<br>DIC-loaded PLGA MP:<br>- Initial burst release of 15%.<br><br>- 1.9% daily release for at least 1 month.<br><br>Insulin-loaded PLGA MP:<br>- Initial burst release of 30%. | DIC-loaded PLGA MP:<br>- BGL at 300-400 mg/dL in the first 10 days.<br><br>- BGL at 100-200 mg/dL from day 10 until day 35.<br><br>Insulin-loaded PLGA MP:<br>- BGL at 300-400 mg/dL in the first 10 days.<br><br>- BGL>300 mg/dL from day 10 until day 35. | The encapsulated DIC was able to reduce the initial burst release and prolong the hypoglycemic effect for 35 days. | (111) |
| **PLA/PEG NP** | Double emulsion solvent evaporation | 182 | 58.5 | AM-induced diabetic New Zealand White rabbits | SC | *In vitro:*<br>Cumulative insulin release of 59%1(85.0 IU) over 10 days. | BGL maintained between 90-140 mg/dL over 8 days. | Able to maintain BGL in a normal physiological range over 8 days. | (80) |
| **PCL NP** | Double emulsion solvent evaporation | 796 | 90.6 | STZ-induced diabetic Wistar rats | SC | - | Reduction of BGL during the first 30 minutes, followed by its maintenance.<br><br>Initial values of BGL after 6h. | Antihyperglycemic activity (with an insulin dose of 2 IU/Kg).<br><br>Non-hepatotoxicity proved. | (96) |

**Table 6: Continued**

| Carrier | Encapsulation method | Mean particle size (nm) | Efficiency of encapsulation (%) | Model | Adm. | Insulin release | Antihyperglycemic activity | Conclusion | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **PEG/PLGA NP** | Modified double emulsion solvent evaporation | 304 | 95.5 | STZ-induced diabetic NIH-Swiss mice | SC | *In vitro:* Initial burst release of 44.6% within 24h. | PEG/PLGA NP:<br>- 12% reduction of BGL within 30 min.<br><br>- Antihyperglycemic activity for 6 days.<br><br>Free insulin:<br>- 58% reduction of BGL within 30 minutes.<br><br>- Antihyperglycemic activity for 3h. | Higher efficiency of encapsulation when comparing with PLGA NP.<br><br>Reduction of initial burst release by reduction of PVA concentration.<br><br>Anti-hyperglycemic effect for 6 days. | (112) |

Abbreviations: Adm.-Administration; AM-alloxan monohydrate; BB-BioBreeding; BGL-blood glucose levels; $CO_2$-Carbon dioxide; DIC-dual interaction complex between insulin and methoxy poly(ethylene glycol)-branched oligoethylenimine-poly (L-histidine); HGC-Hydrophobically modified glycol chitosan; IM-intramuscular; IP-intra-peritoneal; IV-intravenous; MP-Microparticle; MW-molecular weight; NIH-National Institute of Health; NP-Nanoparticle; o/w-oil-in-water; PBS-phosphate buffer saline; PCL-poly-ε-capralactone; PEG-Polyethylene Glycol; PLA-Poly(lactic acid); PLGA-Poly(lactic-co-glycolic acid); PMA-polymethacrylate; PMAA-; PVA-poly(vinyl alcohol); p(CPP:SA)-poly 1,3-bis-(p-carboxyphenoxy) propane-co-sebacic-acid; p(FASA)-poly(fumaric-co-sebacic anhydride); SC-subcutaneous; SD- Sprague-Dawley; SDS-Sodium lauryl sulfate; STZ- streptozotocin; s/o/s-solid-in-oil-in-solid; w/o/w-water-in-oil-in-water

The polymeric NPs and MPs studied in Table 6, which were used to deliver insulin by parenteral route, were characterized by their size, *in vitro* insulin release profile and *in vivo* antihyperglycemic effect. It is noteworthy that in some studies the *in vivo* insulin release profile was also reported. Moreover, the encapsulation method used and the encapsulation efficiency (expressed in percentage and corresponding to the ratio of actual insulin loading to the theoretical loading (64, 80)) was also registered.

In the following, a joint analysis of the aforementioned studies will be presented. It is intended to group the main conclusions by the topics of main encapsulation methods used, release profiles and antihyperglycemic effects observed and, finally the impact of the chosen polymer on the success of the administration of the studied formulation.

The most commonly used encapsulation method was the w/o/w double emulsion solvent evaporation method. However, as already discussed, this method presents some limitations, such as peptide/protein instability due to water/oil interface and shearing forces applied. (60, 65, 77, 89) In order to increase peptide/protein stability and, subsequently its pharmacological effect, different approaches were used in the studies from Table 6.

Firstly, various stabilizers were used. For example, Kumar *et al.* (106) used sodium bicarbonate, trehalose and Pluronic® F68 to stabilize insulin-loaded PLGA NPs. As a result, although a decrease in efficiency of encapsulation was reported, a prolonged antihyperglycemic effect was observed. Also, Naha *et al.* (110) used human serum albumin, sodium bicarbonate, sucrose and mannitol during the formulation process in order to obtain stabilized insulin-loaded PLGA MPs and PLA MPs. Poly(vinyl alcohol, glycerol and zinc oxide were used as stabilizers, in the production of PLA/PEG MPs, by Sheshala *et al.* (64) and zinc oxide stabilized the p(CPP:SA) MPs formulated by Manoharan and Singh. (48)

Secondly, the complexation of insulin with polyeletrolytes was studied. Jiang *et al.* (100) studied the effect of the complexation of insulin with poly(methacrylic acid) (PMAA) before the encapsulation into PLGA MPs. The authors reported a higher encapsulation efficiency for PMAA/PLGA MPs when comparing with PLGA MPs. Also, the bioactivity of insulin was proved by intra-peritoneal injection of the PMAA/PLGA MPs in STZ-induced diabetic Sprague-Dawley (SD) rats.

Lastly, formulation processes alternative to w/o/w double emulsion method were used. For example, solid-in-oil-in-oil emulsion method was used by Han *et al.* (109) to produce NP insulin-loaded PLGA MPs. This method avoids the water/oil interface and, thus

prevents the destabilization of insulin' structure. Besides, it is known that the solid state of a protein maintains its stability, as above mentioned. Encapsulation efficiency higher than 90% was achieved. It should be noted that not only the choice of the method, but also the nanolization of insulin may have contributed to the high efficiency of encapsulation observed. Also, Elvassore *et al.* (99) produced insulin-loaded PEG/PLA NPs by gas antisolvent carbon dioxide ($CO_2$) precipitation. In other words, the solutions of the polymers and the insulin in an organic solvent was atomized through a nozzle into a vessel containing compressed $CO_2$, resulting in its precipitation and subsequently, NPs formation. It is worth noting that despite the bioactivity of insulin was maintained, the efficiency of *in vitro* release was low.

Interestingly, Caliceti *et al.* (98) compared polyphosphazene MPs prepared by different formulation methods: suspension-solvent evaporation, double emulsion-solvent evaporation and suspension/double emulsion-solvent evaporation. The authors reported that the localisation of insulin into the MP was influenced by the formulation method, deeply affecting the insulin release profile. In this respect, MPs formulated by double emulsion-solvent evaporation exhibited the higher initial burst release. However, a longer antihyperglycemic activity was observed for this formulation.

It should be noted that not only w/o/w double emulsion may affect the stability of insulin. Therefore, approaches similar to the above mentioned have been used with other encapsulation methods.

According to the literature, the peptide/protein release from polymeric NPs and MPs depend on different processes: desorption of the peptide/protein bounded to the surface of the particle, diffusion through the polymer matrix/polymer wall (in the case of microspheres/nanospheres or microcapsules/nanocapsules, respectively), polymer degradation and a combination of diffusion and degradation processes. (58, 73, 94, 113, 114)

It should be mentioned that the desorption of the peptide/protein from the surface of the particle is usually associated with an initial burst release, which may compromise the efficiency of the treatment, being also dangerous. (93, 113, 115) Also, the polymer degradation process may disturb the stability of the encapsulated peptide/protein (25, 60, 97, 102) and, thus this topic will be discussed further bellow.

As already mentioned, Table 6 resumes the insulin release profiles studied. It is worth noting that *in vitro* insulin release studies were mostly performed in PBS medium. However, in addition to PBS medium, Shi *et al.* (108) used deionized water to investigate the *in vitro*

release profile of insulin-loaded PLGA NPs. All studies from Table 6 reported a biphasic release profile for insulin-loaded NPs or MPs. In other words, initially a burst release was observed in the first minutes or hours, and then a slower and more prolonged release was achieved.

As the initial burst release of insulin may cause a hypoglycemic episode, it is vital to minimize it. However, it should be noted that high initial burst releases were obtained in some formulations such as the insulin-loaded PLA MPs and PLGA MPs (66% and 71%, respectively) formulated by Ibrahim *et al.* (102) Also, Shi *et al.* (108) reported a high initial burst release (60-65% within 0.5h in PBS medium) for SDS-insulin-loaded PLGA NPs, suggesting that adsorption to the surface of the NPs was the main loading mode.

Different strategies used during the formulation process were able to reduce the amount of insulin released in the initial burst. For example, the use of stabilizers (poly(vinyl alcohol, glycerol and zinc oxide) has not only improved the stabilization of insulin, as already mentioned, but also resulted in the reduction of the initial burst release in insulin-loaded PLA/PEG MP formulated by Sheshala *et al.* (64) Moreover, some modifications in insulin were also formulated. For instance, Hinds *et al.* (101) were able to obtain an initial burst of <1% *in vivo* by PEGylation of the insulin before encapsulation into PLGA MPs. Also, Han *et al.* (109) produced nanolized insulin previously to its encapsulation into PLGA MPs. Compared to commercial insulin-loaded PLGA MPs, NP insulin-loaded PLGA MPs exhibited a decreased initial burst release. It is noteworthy that different morphologies were observed for commercial insulin-loaded PLGA MPs and NP insulin-loaded PLGA MPs. PLGA MPs prepared with nanolized insulin displayed a smooth surface whereas PLGA MPs prepared with commercial insulin exhibited holes on the surface, suggesting that de size of insulin particles influence PLGA MPs' morphology. Also, the authors suggested that the larger size of commercial insulin limits its completely encapsulation and, thus a higher amount of insulin stays adsorbed to the MPs' surface. These differences may explain the alterations observed in the release profile, namely the initial burst release. The encapsulation of the dual interaction complex, resulting from the complexation of insulin with a copolymer consisting on branched oligoethyleneimine, poly(L-histidine) and methoxy poly(ethylene glycol), into PLGA MPs, produced a decrease from 30% to 15% in the initial burst release. (111)

As already mentioned, studies from Table 6 described, after an initial phase of burst release, a second phase characterized by a slow and constant release. For example, Socha *et*

*al.* (79) observed a constant insulin release from heparin coated PCL-PMA NPs for 24h after a high initial burst release of 62% of the insulin within 5 minutes. Another example was reported by Jo *et al. (107)*. The authors described a slow and constant release of insulin from HGC NPs for 120 hours after an initial burst release of 40% of the insulin in the first 12 hours. A prolonged insulin release for weeks and months was also described in several studies. For instance, Sheshala *et al.* (64) reported a constant release of insulin from PLA/PEG MPs for 1 week both *in vitro* and *in vivo*. A slow *in vitro* insulin release from p(CPP:SA) MPs for over 1 month was observed by Manoharan and Singh (48). In fact, after SC injection of insulin-loaded p(CPP:SA) MPs into STZ-induced diabetic SD rats, a release of insulin was detected for 35 days. The most prolonged insulin release was reported by Elvassore *et al. (99)*. The authors described a slow and constant release of insulin from PEG/PLA NPs for over 1500 hours and suggested that the presence of PEG in the NP was responsible for the prolonged duration of the release.

It should be noted that the incomplete release of insulin is usually observed, compromising the efficiency of the carrier. For example, Kim *et al.* (63) described a prolonged insulin release from PLGA MPs for 30 days both *in vitro* and *in vivo*. However, only approximately 50% of the insulin was released. Interaction between the peptide/protein and the polymer has been suggested as one possible cause of the incomplete release often observed. (84)

For evaluation of the antihyperglycemic effect (and *in vivo* insulin release profile), different animal models were used. Most studies were performed in diabetic rats, having DM been induced either by STZ or alloxan monohydrate (AM). Since STZ and AM are known to cause degeneration of the β-cells of islets of Langerhans, which are responsible for insulin production, as aforementioned. (116) Also, it should be noted that Takenaga *et al.* (104) and Furtado *et al.* (103) used Diabetes-Prone BioBreeding rats, which are a model of spontaneous type 1 DM. Since endogenous insulin is not produced in these rats, plasma insulin concentration depends solely on administered insulin.

Most studies administered the encapsulated insulin by SC route. In fact, 14 out of 17 studies that performed *in vivo* evaluations of the antihyperglycemic activity of encapsulated insulin used the SC route as administration route. However, IM, IV and IP routes were also used by Naha *et al.* (110), Socha *et al.* (79) and Jiang *et al.* (100), respectively.

Several formulations showed to be able to reduce blood glucose levels (BGL) after parenteral administration. For example, insulin-loaded PLA/PEG NPs formulated by Tomar *et al.* (80) maintained BGL between 90-140 mg/dL over 8 days after a single SC injection. Jo *et al.* (107) compared the antihyperglycemic activity of insulin-loaded HGC NPs with regular insulin. The authors described a higher reduction of BGL in the first hours post-administration when regular insulin was used. Also, 24 hours post-administration insulin-loaded HGC NPs were able to reduce BGL after oral glucose load. Also, insulin-loaded PLGA MPs produced by Kim *et al.* (63) were able to maintain BGL at 100-200 mg/dL for 55 days whereas hyperglycemic values were observed within 3 days when insulin solution was administered. Promising results were also reported by Haggag *et al.* (112) in a study with PEG/PLGA NPs. Comparing with free insulin, PEG/PLGA NPs showed a smaller reduction in BGL 30 minutes after SC administration (58% reduction when free insulin was used and 12% reduction when PEG/PLGA NPs were used). Besides, PEG/PLGA NPs exhibited a more prolonged antihyperglycemic effect than free insulin (6 days and 3 hours, respectively). Interestingly, SDS-insulin-loaded PLGA NPs prepared by Shi *et al.* (108) showed a slightly better relative bioactivity than free insulin, which may be explained by the inhibition of insulin hexamer formation.

It is noteworthy that despite most studies presented in Table 6 studied the effect of encapsulated insulin after a single dose, multiple doses were also reported. For example, Hinds *et al.* (101) described a multiple dose study consisting on the SC injection of insulin-loaded PLGA MPs into STZ-induced diabetic SD rats at 7-day intervals. The authors reported the achievement of steady state serum insulin levels in the 50-75 µIU/mL range.

It should be mentioned that not all the studies, present in Table 6, that performed *in vivo* evaluations administered the encapsulated insulin. That is to say, the administration of insulin extracted from the polymeric NPs or MPs was reported by Elvassore *et al.* (99) and Han *et al.* (109), respectively. Also, not all the studies that administered the encapsulated insulin performed studies of the release profile of insulin, i.e. in some studies the administration was used only to confirm the maintenance of the bioactivity of insulin. (100, 108) Furthermore, some authors did not perform *in vivo* studies, such as Mukerjee *et al.* (105). Regarding Mukerjee *et al.*' (105) study, it is of note the comparison made between polymeric MPs and NPs formulated by the same method and with the same polymer. The authors reported a controlled release of insulin after a short initial burst release from both

PCL MPs and PCL NPs. However, within 12 hours, a larger amount of insulin was released from PCL MPs.

Another point worthy of being discussed is the influence of the chosen polymer on the stability and subsequently release profile of insulin, namely with regard to polymer degradation as aforementioned.

Different polymers were formulated, as described in studies from Table 6 and the use of different polymers were also compared. For instance, Naha *et al.* (110) compared the insulin release profiles and antihyperglycemic activity of insulin-loaded PLGA MPs and PLA MPs. The authors described similar initial burst release for the studied MPs, however, a faster insulin release was observed for insulin-loaded PLGA MPs for 168h. Also, the IM administration of insulin-loaded PLGA MPs into AM-induced diabetic Wistar rats resulted in a higher reduction of BGL than the administration of insulin-loaded PLA MPs (53.86% and ~40% reduction, respectively).

Nevertheless, the most used polymer in studies from Table 6 was PLGA. The degradation of this polymer, as for other polyesters, results in the accumulation of acidic monomers (lactic and glycolic acids). This acidic environment may cause the denaturation, aggregation and consequently, loss of bioactivity of the encapsulated peptide/protein. (25, 60, 97, 102) Ibrahim *et al.* (102) studied the stability of insulin encapsulated into PLA and PLGA MPs during the release process. Degradation products were observed after 18 days for both PLA and PLGA MPs as well as increased porosity indicating the erosion of the polymers. By that time desamido insulin was present in addition to native insulin.

In order to eliminate the peptide/protein denaturation or aggregation due to the acidic environment inside the polymeric particle, the co-encapsulation of basic additives, such as magnesium hydroxide, calcium carbonate and sodium bicarbonate, has been used. (29, 84, 93, 106) Some studies from Table 6 used this strategy. For instance, Kumar *et al.* (106) prevented the insulin denaturation in the acidic environment inside PLGA NPs by adding sodium bicarbonate during the formulation process.

In addition, polymeric additives were also applied in order to protect insulin from the acidic microenvironment inside PLGA MPs. Park *et al.* (111) synthetized a copolymer consisting on branched oligoethyleneimine, poly(L-histidine) and methoxy poly(ethylene glycol). This copolymer (methoxy poly(ethylene glycol)-branched oligoethylenimine-poly (L-histidine), POH)) was complexed with insulin and the resulting dual interaction complex was

encapsulated into PLGA MPs. The encapsulation efficiency was found to be higher for DIC-loaded PLGA MPs than for insulin-loaded PLGA MPs. Also, the DIC-loaded PLGA MPs were able to prolong the antihyperglycemic activity for 35 days.

Furthermore, the molecular weight and the copolymer ratio may also affect the pharmacokinetic properties of the formulation. For example, Manoharan and Singh (48) formulated insulin-loaded p(CPP:SA) MPs with different copolymers ratios: 20:80, 40:60 and 50:50. As a result of an increased hydrolysis and faster degradation of SA bonds, the highest initial burst release was observed for MPs formulated with the copolymer ratio of 20:80. CPP:SA 50:50 presented the lowest initial burst release and a more prolonged insulin release than the other formulations. Also, two different copolymer ratios of PLGA (50:50 and 85:15) were used by Kumar *et al.* (106) in the formulation of insulin-loaded PLGA NPs. The authors described a smaller mean particle size and a more sustained antihyperglycemic effect when the copolymer ratio was 85:15. In addition, Han *et al.* (109) studied the effect of the weight of PLGA in the properties of insulin-loaded PLGA MPs. However, when the weight of the polymer was varied (10k, 25k and 90k PLGA were used), no significant influence on encapsulation efficiency was observed and only a slight difference was seen on the insulin release profiles.

Finally, it is interesting to note that two different approaches to improve insulin therapy were simultaneously used: PEGylation and encapsulation. Hinds *et al.* (101) encapsulated PEGylated insulin into PLGA MPs and showed that the combination of PEGylation and encapsulation was able to release insulin in a constant way for over 16 days. In fact, according to the literature, the combination of these two strategies is usually advantageous. For example, PEGylation of the peptide/protein often results in increased stability, which is often a problem during the formulation and release processes for encapsulated peptides/proteins, as aforementioned. Besides, PEGylation of the peptide/protein was associated with an increased solubility in both aqueous and organic solvents, allowing the usage of oil-in-water single emulsion (as used by Hinds *et al. (101)*), which is a simpler encapsulation method than w/o/w double emulsion. Moreover, PEGylation of the peptide/protein contributes to the reduction of adsorption of the peptide/protein to the polymer as well as the decrease of the initial burst release of the peptide/protein. It should also be noted that not only microencapsulation technique is improved by the use of PEGylated peptides/proteins instead of unmodified peptides/proteins, but also PEGylation

technique is improved by encapsulation, namely by the prolongation of the sustained release of the peptide/protein. (115) Nevertheless, it is noteworthy that using two different approaches simultaneously means that the development time and costs increase when comparing to the use of one of the strategies alone. Therefore, it may be a burden for a pharmaceutical company. (115)

# 4. CONCLUSION

The epidemic proportions reached by DM makes it urgent to improve its therapy in order to be able to achieve a better control of BGL in diabetics, preventing the development of complications associated with high BGL. (1) For insulin-dependent diabetics insulin therapy is the only option. Since the beginning of insulin therapy in 1922 several improvements were made, especially with the development of insulin analogues. (10, 22, 33, 34) However, nowadays insulin therapy still presents some drawbacks, such as the risk of hypoglycaemia, weight gain and poor patient compliance. (15, 23, 47) For that reason, the search for a better insulin therapy has to continue. In this regard, NT presents some possibilities, namely the controlled delivery of insulin through its encapsulation into polymeric particles.

This thesis summarized the conclusions of several studies that tested the encapsulation of insulin into polymeric NPs and MPs and subsequent parenteral administration. It was concluded that despite these strategy has potential to improve the current insulin therapy, some issues need to be solved. The initial burst release exhibited may cause hypoglycemic episodes and, so it is vital to reduce it. Also, the incomplete release of insulin may compromise the efficiency of the therapy. The stability of insulin both in the formulation and release processes is another concern. Therefore, the usage of polymers and formulation methods that do not affect insulin stability is vital. (63, 115) On the other hand, the encapsulation of insulin into polymeric particles were able to prolong insulin action. Therefore, the frequency of insulin injection, which contributes significantly for poor patient compliance, is reduced. (90) Consequently, a better control of BGL is possible, reducing the risk of development of complications associated with hyperglycemic values. Furthermore, the encapsulation of insulin into polymeric NPs and MPs is particularly important for the maintenance of basal insulin levels as it allows a slow and constant release of insulin for a longer period of time, which better mimics the normal physiological process. Also, as insulin is released at a constant rate, the excessive tissue and muscle exposure to insulin seen after the injection of unencapsulated insulin is avoided. (103)

It should be noted that the ideal formulation for the post-prandial administration of insulin, should present a pulsatile release. That is to say, insulin release would occur in response to the increase of BGL. (97, 117)

Another point worthy of being mentioned is the study of the potential improvement of the current insulin therapy by other strategies, such as the administration of encapsulated insulin by non-invasive routes. In fact, several studies have been performed using pulmonary, oral, transdermal routes of administration, among others. (47, 95, 118) However, despite many attentions have been focused in these strategies, the parenteral route is the most used for insulin administration as for other peptides/proteins. (71, 93)

In conclusion, despite some issues are still to be solved, the encapsulation of insulin into polymeric particles is able to release insulin in a way closer to the physiological process than the current insulin therapy. (103)

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