Universidade do Algarve

Faculdade de Ciências e Tecnologia

Lipid nanoparticles for therapeutic proteins delivery

Fatumata Ramadana Gomes Seck

Dissertação para obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho realizado sob a orientação:
Professor Doutor Pedro Ricardo Martins Lopes da Fonte

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Live as if you were to die tomorrow. Learn as if you were to live forever.

Mahatma Gandhi

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Abstract

Therapeutic proteins are bioactive compounds used for the treatment and prevention of several diseases. These compounds are usually well-tolerated, present a high specific activity, few adverse reactions, and a wide range of applications. Nevertheless, they also present physicochemical instability, with susceptibility to suffer degradation. The use of nanocarrier systems protects the protein structure, improve its bioavailability and enhance its sustained or controlled release. There has been an emerging interest in lipid nanoparticles as carriers for drug delivery. Solid lipid nanoparticles (SLN) are considered the first-generation of lipid nanoparticles, composed of a solid lipid matrix of one or more biocompatible and biodegradable lipids, solid at both room and body temperature. Nanostructured lipid carriers (NLC) are the second generation of lipid nanoparticles, on which the solid lipid matrix is replaced by a blend of liquid and solid lipid. The incorporation of a liquid lipid increases the imperfections in the matrix core which allows an increased encapsulation efficiency and decreased expulsion of the encapsulated drugs during storage. Nevertheless, the application of the NLC for the encapsulation of therapeutic proteins is not well established yet and it urges the need to optimize production methods that do not compromise the protein structure during the encapsulation process. We have optimized the production of an insulin-loaded NLC formulation achieving a particle size of about 200 nm, zeta potential of -18 mV and more importantly, an encapsulation efficiency of about 85% and loading capacity of 11%, which are promising features for different applications. Therefore, the objective of this work was to address the use of lipid nanoparticles for therapeutic proteins delivery. Thus, using insulin as a model protein, it was developed a production method to encapsulate therapeutic proteins into NLC. It is foreseen the opening of a new paradigm in the delivery of therapeutic proteins using NLC.

Keywords: Solid lipidic nanoparticle; Nanostructured lipid carrier; Therapeutic protein; Delivery system; Insulin; Encapsulation; Drug delivery.

Resumo

As proteínas terapêuticas são compostos bioativos usados no tratamento e prevenção de várias doenças. Estes compostos são geralmente bem tolerados, apresentam atividade altamente específica, poucas reações adversas e uma ampla área de aplicações. No entanto, também apresentam instabilidade físico-química e suscetibilidade a sofrer degradação. O uso de sistemas de transporte nanoestruturados protege a estrutura da proteína, melhora a sua biodisponibilidade e aumenta a sua libertação controlada ou sustentada. O interesse no uso de nanopartículas lipídicas em sistemas de administração de fármacos tem vindo a aumentar. As nanopartículas lipídicas sólidas (NLS) são a primeira geração, compostas por uma matriz lipídica sólida de um ou mais lípidos biocompatíveis e biodegradáveis, sólidos à temperatura ambiente e corporal. Os transportadores lipídicos nanoestruturados (TLN) são a segunda geração, nos quais a matriz lipídica sólida é substituída por uma mistura de lípido líquido e sólido. A incorporação de um lípido líquido aumenta as imperfeições na matriz o que permite uma maior eficiência de encapsulações e reduz a expulsão da proteína durante o período de armazenamento. Contudo, a aplicação de TLN para encapsulação de proteínas terapêuticas não está ainda bem estabelecida e urge a necessidade de se otimizar métodos de produção, que não comprometam a estrutura da proteína. Otimizou-se a produção de uma formulação de insulina encapsulada em TLN, alcançando-se um tamanho de partícula de cerca de 200 nm, zeta potencial de -18 mV e mais importante, eficiência de encapsulação de 86% e capacidade de encapsulação de 11%, que são características promissoras para diferentes aplicações. Assim, o objetivo deste trabalho foi descrever o uso de nanopartículas lipídicas para o transporte de proteínas terapêuticas. Deste modo, usando-se a insulina como proteína modelo, foi desenvolvido um método de produção para a encapsulação de proteínas terapêuticas em TLN. Prevê-se abrir um novo paradigma no transporte de proteínas terapêuticas usando TLN.

Palavras-chave: Nanopartícula lipídica sólida; Transportador lipídico nanoestruturado; Proteína terapêutica; Sistema de administração; Insulina; Encapsulação

Resumo alargado

A introdução de proteínas para fins terapêuticos é uma das maiores conquistas da ciência moderna, e a sua aplicabilidade nas diferentes áreas da medicina tem estado a evoluir continuamente, alterando o paradigma de tratamento em várias áreas terapêuticas.

As proteínas terapêuticas apresentam várias vantagens comparativamente aos fármacos de síntese química, sendo capazes de obter resultados que de outra forma não seriam possíveis de alcançar com fármacos de síntese química. Em primeiro lugar, apresentam alta especificidade e cobrem uma vasta área de aplicações que não seria possível recriar com compostos químicos. Como a sua ação biológica é altamente específica, o risco de interferirem com processos biológicos e provocarem reações adversas é significativamente menor. De modo geral, são também muito bem toleradas porque muitas das proteínas usadas para fins terapêuticos são produzidas endogenamente. No entanto, apesar de todas as vantagens das proteínas terapêuticas, a sua administração ainda representa um desafio, essencialmente devido aos seus problemas de imunogenicidade, fraca biodisponibilidade devido à instabilidade física e química e consequente rápida eliminação no soro sanguíneo, desafios de produção e dificuldade em atravessar membranas celulares. Devido à sua rápida eliminação, são normalmente administradas parentericamente, em altas e repetidas doses para as manter em concentrações terapêuticas durante o desejado período de tempo, o que é um processo doloroso e mal tolerado pelos doentes, diminuído a adesão à terapêutica.

Existem várias abordagens para ultrapassar os desafios associados às proteínas terapêuticas, que podem ser agrupadas em quatro categorias: manipulação de aminoácidos, bioconjugação, modificação pós translação e distribuição mediada por transportadores. A técnica de manipulação de aminoácidos consiste em inserir, deletar ou alterar um ou vários aminoácidos na cadeia proteica, o que se provou que reduz a imunogenicidade e a clivagem proteolítica *in vivo*. O princípio de base das abordagens de bioconjugação, é que durante o metabolismo hepático, as proteínas são geralmente captadas pelos hepatócitos, num processo mediado por recetores. Depois disso, são degradadas em lisossomas por enzimas e eliminadas da circulação. No entanto algumas proteínas endógenas conseguem evitar o metabolismo hepático, imitando a reciclagem mediada por recetor das proteínas endógenas. Desse modo, foi observado que ao ligar-se as proteínas terapêuticas a certas proteínas endógenas, como a albumina e a imunoglobulina, a reciclagem acoplada por recetor ajuda a redirigir as proteínas

terapêuticas novamente para a circulação, podendo ser usado como uma estratégia para evitar a degradação enzimática e estender o tempo de semivida em circulação. Uma das estratégias usadas para reduzir a clearance renal consiste em aumentar o tamanho e peso molecular da proteína terapêutica. Neste sentido, a modificação pós translação, consiste em unir a proteína a polímeros, de origem natural ou sintética, para aumentar o volume hidrodinâmico, prevenir a rápida clearance renal e desse modo aumentar o tempo de semivida da proteína. Os sistemas de transporte são usados com o propósito de proteger a proteína contra degradação *in vivo*. Além disso, alguns transportadores também permitem modelar a administração controlada e dirigida das proteínas terapêuticas.

A nanotecnologia permite desenvolver partículas, aparelhos e sistemas dentro da escala nano, tendo vindo a ganhar importância no desenvolvimento de fármacos, com o potencial de remodelar a área terapêutica e desenvolverem-se agentes terapêuticos mais eficientes, mais específicos, menos tóxicos e com ação dirigida. As nanopartículas podem ser idealizadas de modo a adquirirem propriedades física e químicas especiais, que as permitam interagir com células e tecidos ao nível molecular e atómico, permitindo uma nova gama de possibilidades com o ambiente biológico, dirigir a sua ação a células e recetores celulares, libertação controlada e influenciar os mecanismos moleculares da doença. Quando aplicadas aos sistemas de distribuição de proteínas terapêuticas, as nanopartículas permitem ultrapassar os desafios associados à distribuição de proteínas terapêuticas previamente mencionadas.

Nos últimos anos, as nanopartículas lipídicas têm vindo a ser estudadas para uso em sistemas de administração de fármacos, essencialmente na tentativa de ultrapassar os problemas e melhorar as características das proteínas terapêuticas, protegendo-as da degradação *in vivo*, permitindo uma libertação controlada, modificando a bio distribuição e melhorando a ação dirigida, solubilidade e biodisponibilidade. O uso de lípidos no desenvolvimento de sistemas de distribuição iniciou-se com as vesículas fosfolipídicas, designadas de "lipossomas" em 1965 pelo Prof. A.D. Bangham. Os lipossomas são sistemas vesiculares esféricos, compostos por uma ou várias bicamadas, que aprisionam um pequeno volume da fase aquosa. Foram primeiramente introduzidas no mercado cosmético em 1986 e posteriormente em produtos farmacêuticos no fim de 1980. Alguns anos depois, foi desenvolvida uma nova geração de nanopartículas lipídicas, com uma matriz sólida à temperatura ambiente, as nanopartículas lípicas sólidas e os transportadores lipídicos nanoestruturados. As nanopartículas lipídicas sólidas são a primeira geração de nanopartículas lipídicas, compostas por uma matriz lipídica sólida de

um ou mais lípidos biodegradáveis e biocompatíveis. Os transportadores lipídicos nanoestruturados são a segunda geração e foram desenvolvidas para ultrapassar as desvantagens das nanopartículas lipídicas sólidas, relacionadas com a perfeita cristalização da sua estrutura matricial, responsável pelo baixa capacidade de encapsulação das nanopartículas lipídicas sólidas e expulsão da proteína encapsulada durante o período de armazenamento. Ao apresentar uma matriz composta por dois lípidos espacialmente diferentes, a matriz dos transportadores lipídicos nanoestruturados apresenta imperfeições que aumentam a capacidade de encapsulação, acomodam melhor a proteína terapêutica, evitando a sua expulsão durante o período de armazenamento e permitem melhores propriedades de libertação sustentada, quando comparadas com nanopartículas lipídicas sólidas.

Tanto as nanopartículas lipídicas sólidas como os transportadores lipídicos nanoestruturados usam os mesmos métodos de produção que se encontram muito bem estabelecidos, mas que por norma não são indicados para a encapsulação de proteínas terapêuticas porque utilizam frequentemente temperatura e/ou altas pressões, que são condições de stress que podem danificar a estrutura tridimensional da proteína e comprometer a sua atividade terapêutica. Assim, é necessário adaptar esses métodos tecnológicos, de forma a que seja possível a encapsulação de proteínas terapêuticas de forma efetiva.

O objetivo deste trabalho foi apresentar uma visão global da aplicação de nanopartículas lipídicas para a administração de proteínas terapêuticas. Mais importante, usando a insulina como proteína terapêutica modelo, desenvolveu-se um método de produção para a encapsulação de proteínas terapêuticas em transportadores lipídicos nanoestruturados, sem comprometer a sua estrutura e biodisponibilidade.

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List of abbreviations and acronyms

ADME - Absorption, distribution, metabolism and excretion

AE – Association efficiency

Ala - Alanine

BCC - Business Communications Company

BSA – Bovine serum albumin

CNT - Carbon Nanotubes

DNA - Deoxyribonucleic Acid

EE – Encapsulation efficiency

FDA – Food and Drug Administration

GCSLN - Gel core solid lipid nanoparticles

GRAS - Generally recognized as safe

HLB - Hydrophilic-lipophilic balance

HPH – Hot pressure homogenization

LC - Loading capacity

MP – Melting point

NLS - *Nanopartículas lipídicas sólidas*

NP - Nanoparticle

NLC – Nanostructured lipid carriers

NNI - National Nanotechnology Initiative

PdI - Polydispersity index

PEG – Polyethylene glycol

pI – Isoelectric point

SLN – Solid lipid nanoparticles

The - Threonine

TLN - *Transportadores lipídicos nanoestruturados*

USA – United States of America

Val - Valine

VB12 - Vitamin B

1. Introduction

The introduction of proteins as therapeutics is one of the major achievements of modern science, and their application has been continuously evolving, reshaping several fields of medicine. Proteins as therapeutics present several advantages when compared with synthetic drugs, being able to obtain results that otherwise would not be possible to achieve with synthetic drugs. Nevertheless, they also present limitations that impose difficulties in the drug development process and its use as therapeutics because of immunogenicity issues, poor oral bioavailability, physical and chemical instability, rapid serum clearance, susceptibility to suffer enzymatic degradation, and difficulty to permeate membranes (1).

Nanotechnology allows to develop of particles, devices and systems within the nanoscale and has been gaining increased importance in drug development, with the potential to remodel the medical treatment and achieve therapeutics more efficient, more specific, less toxic and with targeted delivery (2). The nanomaterials can be designed to acquire unique physical and chemical properties, allowing it to interact with cells and tissues at a molecular and atomic level, ensuring a new range of possibilities with the biological environment, targeting cells and cell-surface receptors, controlling drug release and multiple drug administrations, and influence the molecular mechanisms of the disease. When applied for drug delivery of therapeutic proteins, nanoparticles allow to overcome the therapeutic proteins delivery challenges (3,4).

In the last years, lipid nanoparticles have been studied as drug delivery systems, essentially as an attempt to overcome the problems and improve the characteristics of therapeutic proteins, protecting them from degradation *in vivo*, allowing controlled drug release, modifying biodistribution, and enhancing targeted delivery, solubility and bioavailability. The use of lipids in the development of delivery systems started with phospholipid vesicles rediscovered as “liposomes” in 1965 by Prof. A.D. Bangham. Liposomes are spherical vesicular systems, composed of one or multiple bilayers entrapping a small volume of the aqueous phase, firstly introduced in the cosmetic market in 1986, and after in pharmaceutical products at the end of the 1980s (5–7). A few years later, it was developed a new generation of lipid nanoparticles, with a solid matrix at room temperature, the SLN and the NLC. The SLN are the first generation of lipid nanoparticles, composed of a solid lipid matrix of biodegradable and biocompatible lipid

or blend of lipids. The NLC are the second generation and were developed to overcome the disadvantages of the SLN related to their perfect crystallization matrix structure, which is responsible for the SLN low loading capacity and expulsion of the encapsulated drug during storage. By presenting a matrix composed of two spatially different lipids, the NLC matrix presents imperfections that increase their loading capacity, better accommodate the encapsulated drug, avoid drug expulsion during storage and allow better sustained released properties compared to SLN.

Both SLN and NLC use the same production methods which are very well established but usually not suited for the encapsulation of therapeutic proteins because they often apply temperature and/or high pressures that can damage the tri-dimensional structure of the protein and compromise its therapeutic activity (1,8).

The objective of this work is to make an overview of the application of lipid nanoparticles for the delivery of therapeutic proteins. More importantly, using insulin as a model therapeutic protein, it was objective to develop a production method for the encapsulation of therapeutic proteins into NLC, without compromising its structure and bioavailability.

1.1. Therapeutic proteins

Proteins were recognised in the eighteenth century as biological molecules with specific proprieties, in particular, the capacity to coagulate when treated with heat or acid. The term “protein” was proposed in 1838 by Jöns Jakob Berzelius (9). Currently, there are over 250 proteins used clinically for different purposes from prophylaxy as is the case of some vaccines, to clinical treatment of metabolic diseases or even cancer (10,11).

Therapeutic proteins are, by definition, macromolecular drugs produced by biotechnology, using live organisms and their active compounds (12). The best example in the production and use of therapeutic proteins is the history of insulin in the treatment of diabetes *mellitus*. Insulin is an anabolic heterodimer composed of two chains, the A-chain with 21 residues and the B-chain with 30 residues, both linked by two disulfide bonds and an additional intrachain disulfide bond present in the A-chain, as shown in Figure 1.1 (13). In 1922 insulin was first purified from bovine and porcine pancreas and used for the treatment of diabetic patients, emerging as a life-saving treatment (14). Nonetheless, with the widespread use of this protein, some problems came to light: the limited availability of animal pancreases for purification of insulin which wouldn’t be

sufficient for the daily treatments of patients, the cost associated with the process and the immunological reactions developed by some patients. To solve these problems and take advantage of the advances in bioengineering, the human insulin gene was isolated and *Escherichia coli* was engineered to express the human insulin, using recombinant deoxyribonucleic acid (DNA) technology. By growing large quantities of this bacteria, the large-scale production of human insulin was accomplished and, in 1982, recombinant insulin was approved by the Food and Drug Administration (FDA), representing one of the biggest achievements of modern healthcare science (14).

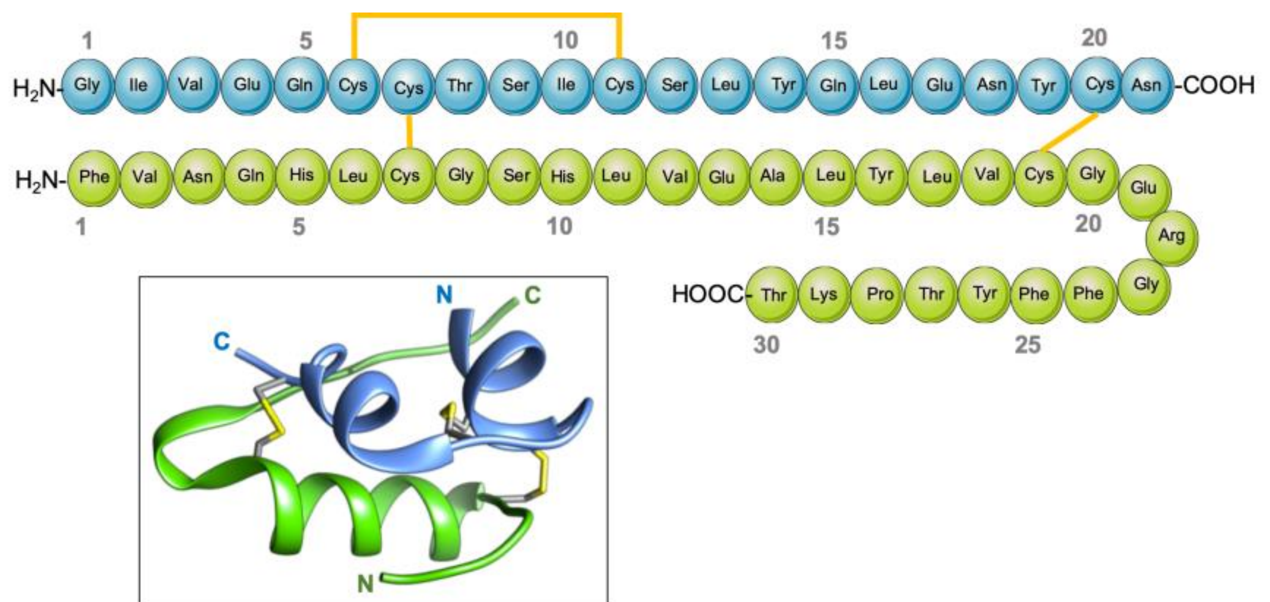

Figure 1.1. Schematic primary structure of human insulin and its 3D structure. The A-chain is shown in blue, the B-chain in green and the intra- and into-chain disulfide bonds at yellow. The porcine insulin only varies in the B30 position where the threonine (Thr) is replaced by an alanine (Ala). The bovine insulin contains that same substitution as well as Ala in position A8 and valine (Val) in position A10 (15). *Reprinted with permission from* (16)

Recombinant DNA technology established on an industrial scale has dramatically escalated the number of biotechnology drugs approved and under investigation. According to the numbers from 2018, therapeutic proteins alone, excluding peptides and genetic-based ones, corresponded to 199 entities in the United States of America (USA), Europe, and Canada. Moreover, according to a study led by the Business Communications Company (BCC) Research, the global market for bioengineered protein drugs in 2016 was evaluated at $172.5 billion and it is expected to reach $228.4 billion by 2021. In terms of the annual growth rate, in 2016 was $39.8 billion and it is predicted

to reach $40.2 billion in 2021, which is about 10% of the ethical pharmaceutical market (17,18). The increasing number of protein therapies that have been used for a wide range of applications include hormones, enzymes, clotting factors, antibodies and may be classified according to their pharmacologic activity or grouped into molecular types as shown in Table 1.1 (12,14,19–22).

Table 1.1. Classification of therapeutic proteins by pharmacologic activity. *Adapted with permission from* (14,23)

Pharmacologic activity		**Therapeutic proteins**
Group I: Enzymatic or regulatory activity	Ia: Replace a deficient or abnormal protein	Insulin, Factor VIII, lactase
	Ib: Augment an existing pathway	Erythropoietin, Human follicle-stimulating hormone (FSH), Alteplase
	Ic: Provide a novel function or activity	Botulinum toxin type A, Rasburicase, Bivalirudin
Group II: Special targeting activity	IIa: Interfere with a molecule or organism	Trastuzumab, Adalimumab, Omalizumab
	IIb: Deliver a payload	Denileukin diftitox, Gemtuzumab ozogamicin, tositumomab
Group III: Vaccines	IIIa: Protecting against a deleterious foreign agent	HPV vaccine, OspA
	IIIb: Treating autoimmune diseases	Anti-Rhesus (Rh) immunoglobulin G
	IIIc: Treating cancer	In clinical trials Melanoma cancer vaccine (Phase 2), NeuVax (Phase 2/3), CYT004-MelQbG10 (Phase 2)
Group IV: Diagnostics		Secretin, Arcitumomab, Hepatitis C antigens

Therapeutic proteins have several advantages over synthetic drugs. Firstly, they present high specificity and cover a wide range of functions that cannot be mimicked by chemical compounds. Since their biological action is very specific, the risk of interfering with biological processes' and causing adverse reactions is significantly lower. In general, they are also very well tolerated because the body naturally produces many of the proteins that are used as therapeutics. From a financial perspective, they are also more appealing when compared to synthetic drugs for two very important reasons. The first reason is related to the fact that the clinical development and approval time of protein drugs is more than one year faster than for synthetic drugs. The results from a 2003 study showed that the average clinical development and approval time for 33 therapeutic proteins approved between 1980 and 2002 was more than 1 year faster than for 294 small-molecule drugs approved during the same period (24). A more recent study of clinical drug development success rates from 2021 analysed 6151 successful phase transitions during the 2011–2020 period, concluding that it took in average 10,3 years for a therapeutic protein to reach the market, including 2,3 years at Phase I, 3,6 years at Phase II, 3,3 years at Phase III, and 1,3 years at the regulatory stage (25). In Figure 1.2 it is represented the duration of the phases of drug development for therapeutic proteins by disease and in Figure 1.3 the same timeline for synthetic drugs. Moreover, due to their singularity in terms of form and function, companies can obtain far-reaching patent protection (14).

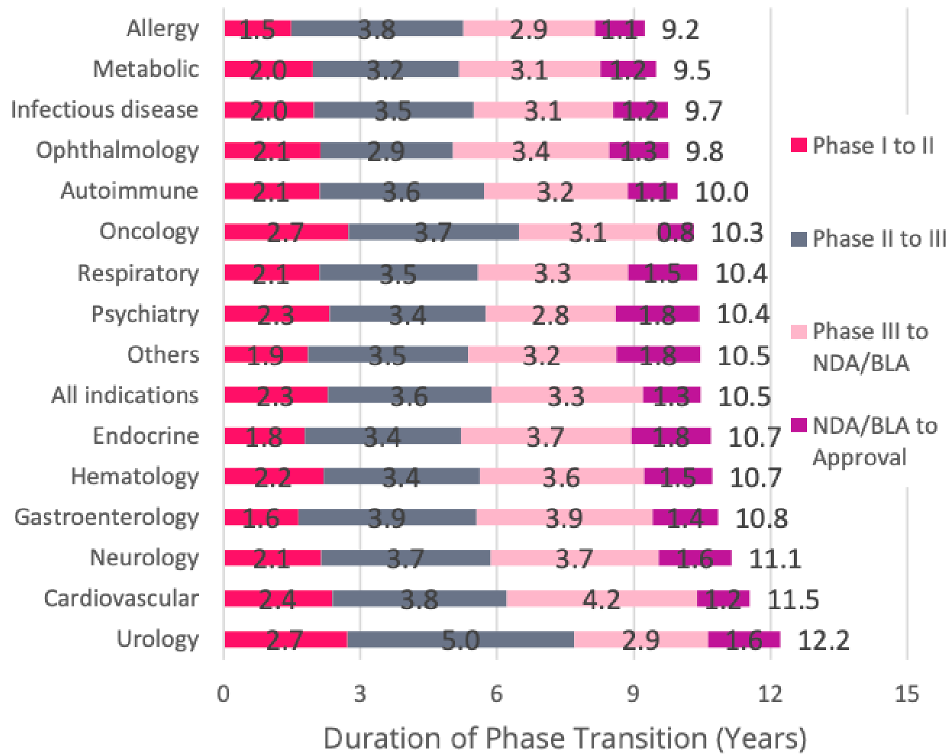

Figure 1.2. Timeline for drug development for therapeutic proteins by disease. These results are based on 6151 successful phase transitions in the 2011–2020 period. *Reprinted with permission from* (25)

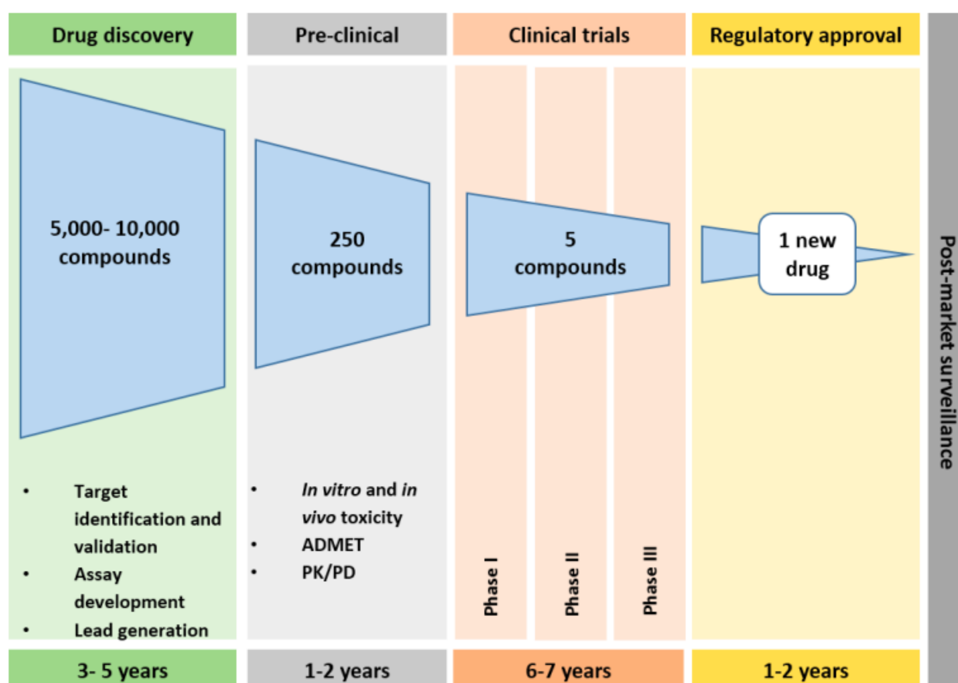

Figure 1.3. Drug discovery and development timeline for synthetic drugs. It takes on average approximately 15 years for a synthetic drug to reach the market. *Reprinted with permission from* (26)

Nonetheless, as shown in Table 1.2, despite all the advantages, the administration of protein drugs still represents a challenge, mainly due to their immunogenicity problems, poor bioavailability due to their physicochemical instability and consequent fast degradation in serum, production challenges and difficulty to permeate membranes as the gastrointestinal membrane. Since therapeutic proteins suffer rapid degradation in serum and fast elimination, they are usually administrated parenterally in high and repeated doses to maintain it in therapeutic concentrations for the desired time, which is painful and not well tolerated by patients, decreasing patient compliance to treatment. Furthermore, due to their short residence period in blood before suffering renal clearance and enzymatic degradation, it urges the need to administer high doses to reach therapeutic concentrations for the desired period. This administration profile creates a variable concentration of the therapeutic protein preceded by a high initial peak that ultimately leads to side effects (19,27). To diminish those side effects and attend to their narrow therapeutic ranges, several approaches have been developed and tested to extend the therapeutical protein's half-life in circulation. By extending the protein’s half-life, both of the problems mentioned would be addressed, maintaining the therapeutical concentrations with lower doses (19,27,28).

Table 1.2. Advantages and disadvantages of therapeutic proteins. *Adapted from* (14,17)

Advantages	**Disadvantages**
High specificity	Immunogenicity problems
Wide range of application	Poor oral bioavailability
Low incidence of adverse reactions	Physical and chemical instability
Well tolerated	Rapid clearance
High potency	Enzymatic degradation
High chemical and biological diversity	
Low toxicity	Difficulty to permeate membranes
Low accumulation in tissues	

If it is true that therapeutic protein is one the fastest growing class of drug molecules, is also true that developing strategies to overcome the obstacles imposed by their administration problems are crucial to increase the number of therapeutic proteins reaching the pharmaceutical market (11,19).

1.1.1. Delivery challenges of therapeutic proteins

As mentioned previously, therapeutic proteins have delivery challenges that compromise their therapeutic effect and limit their administration routes. From those, their immunogenicity, short half-life, isoelectric point (pI) and modification of the protein charge, structural stability and membrane permeation and glycosylation profile are the most impactful and are discussed in this section.

1.1.1.1 Immunogenicity

The development of therapeutic proteins was accompanied by the expectation that the same as the "self" derived proteins, they would avoid immunogenicity due to central tolerance. Unfortunately, this idea has been proven to be flawed, with several examples of recombinant proteins that stimulate host immune responses, originating anti-therapeutic antibody response. The generation of these anti-therapeutic antibodies involves stimulation of multiple components of the immune system, both adaptative and non-adaptative immune responses which means that immunogenicity of protein therapeutics cannot be imputed to a single factor. This is a serious and concerning problem since these responses can have a neutralizing or non-neutralizing effect on the protein. This can reduce the protein half-life or trigger allergic reactions if the therapeutical is non-endogenous alike. But if the protein drug has antigenic similarities with an endogenous protein, then a neutralizing antibody response can cross-react with the endogenous protein, resulting in scenarios of morbidity and mortality. Moreover, the immunogenicity of protein therapeutics is remarkably hard to predict before clinical trials because the traditional animal models used for synthetic drugs are of limited application for therapeutic protein drugs (29).

There is also a relationship between aggregated proteins and enhanced immunogenicity, with studies showing this correlation in a variety of models (29). Protein aggregation is defined as the self-association of monomers either in their native or partially unfolded forms, a process that can occur during the life of a therapeutic protein

induced by a wide range of factors like temperature, mechanical stress, freezing and thawing (30–34). According to a study developed by Braun et al., the IFN-alpha protein aggregates (IFN-alpha-IFN-alpha and human serum albumin (HSA)-IFN-alpha aggregates) presented considerable higher immunogenicity than the IFN-alpha monomers. The results from a study in 2011, also showed augmented immunogenicity of aggregated rhIFNβ-1a in transgenic mice (35).

1.1.1.2. Short half-life

Pharmacokinetics is, by definition, the study of the movement of xenobiotics (drugs/compounds/new chemical entities) within the body after administration, being affected by four distinct, yet interrelated processes: absorption, distribution, metabolism, and excretion (ADME) (36). The efficacy of therapeutical proteins is significantly affected by their pharmacokinetic properties as their plasma half-life (37).

Since most of the endogenous proteins activity resembles with hormones activity, they frequently present fast serum elimination, which is desirable from the hormonal regulation point of view. Nonetheless, therapeutic proteins are nearly completely metabolized through the same catabolic pathways as endogenous or dietary proteins, which leads to also fast clearance, or nonmetabolic elimination pathways as renal or biliary excretion.

Depending on the protein size, renal filtration can be determinant for the protein half-life in serum. Two main factors affect kidney filtration: protein size and hydrophobicity. The kidney filtration cut off size for a peptide is < 70 kilodalton (kDa) which means that peptides smaller than that will easily get cleared by the kidneys, which also means that as the hydrodynamic radius of the protein increases, the renal clearance decreases (38–40). Yet, there is a wide diversity of therapeutic proteins including monoclonal antibodies, enzymes, hormones, growth factors and cytokines, each one with specific average molecular weights, making this class very heterogeneous in terms of the range of molecular weights.

Deamidation occurs when the amide groups of asparaginyl or glutaminyl residues are hydrolyzed to a free carboxylic acid because of susceptibility to extreme pH conditions. This is also responsible for the short half-life of therapeutic proteins.

Yan et al. (41) studied the impact of the deamidation rate of asparagine in the protein structural features. Different stress conditions were employed, using extreme pH

(8.5) and high temperature stress (37 °C) to identify the asparagine sites sensitive to deamidation in IgG mAbs. The results showed that difference in asparagine deamidation rate could be due to structure conformation, structure flexibility and solvent accessibility (42).

1.1.1.3. Isoelectric point (pI) and modification of the protein charge

Globular proteins are actively adsorbed to hydrophobic and hydrophilic interfaces as production tanks, glass vials or processing components, which significantly influence their pharmacokinetics and biodistribution, leading in some cases to aggregation of the therapeutic protein and eventually to a decrease in concentration. The pI, which is the pH of a solution at which the protein maintains zero net charge has a considerable influence on the adsorption of proteins to hydrophilic and charged surfaces. According to a therapeutic protein local physiological environment, the overall charge of the protein can vary which means that according to the strength of the interaction, the therapeutic proteins may be adsorbed (40,42).

A study on the characterization of protein adsorption onto silica nanoparticles, highlighted the impact of isoelectric interactions on globular proteins Lyz and ß-Lg onto negatively charged silica nanoparticles. In both cases, it was verified that for low pH values, the competition between the attractive protein-surface and the repulsive protein-protein interactions limited the adsorption to one monolayer of the protein's molecules. For pH values closer to pI the protein-protein interactions were less relevant which extended the adsorption significantly above one monolayer (43).

1.1.1.4. Structural stability and membrane permeation

Therapeutic proteins present high susceptibility to suffer chemical and physical degradation. Physical instability refers to events that lead to conformational changes in the protein structure that includes protein unfolding, aggregation, precipitation and adsorption to the surface. Chemical instability, on the other hand, is related to the formation or destruction of covalent bonds within the protein molecule, which modifies the primary structure of the protein and therefore its structure and eventually its bioactivity and therapeutic effect. The most frequent causes for chemical instability include deamidation, oxidation and cystine destruction or disulfide exchange. In Figure

1.4 is represented the different physical and chemical instability sources of protein therapeutics (44,45).

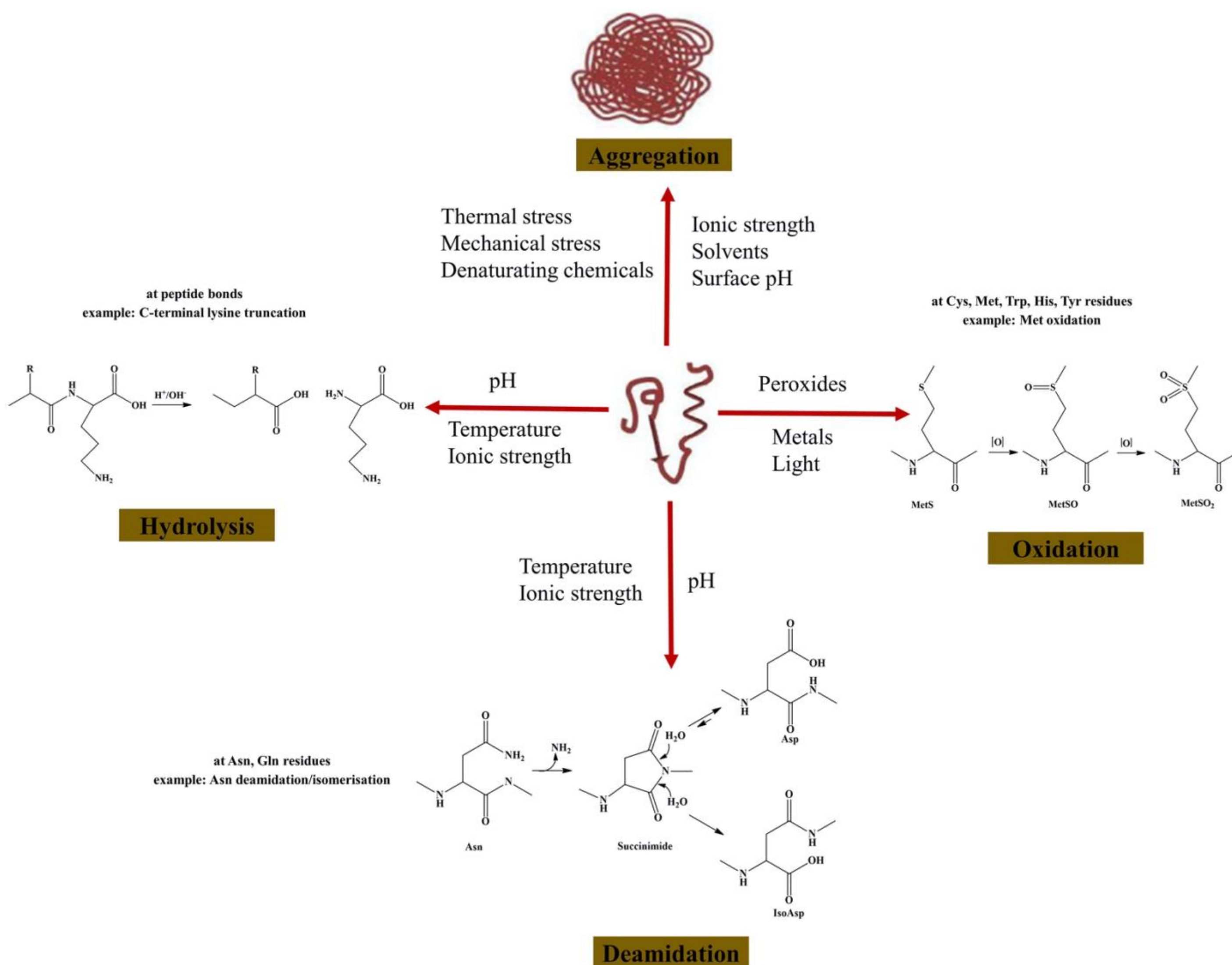

Figure 1.4. Physical and chemical instability sources of therapeutic proteins. *Adapted from* (45)

Therapeutic proteins are usually administered parenterally (intravenously, subcutaneously or intramuscularly) due to their high susceptibility to suffer proteolysis in the gut and their difficulty to permeate membranes. Apart from drugs administrated intravenously, all drugs administered by other routes will have to permeate membranes to be absorbed. The gastrointestinal tract (GIT) is the most important site for drug absorption, since oral administration is the preferred route of administration. GIT permeation rate of compounds is dependent on the intestinal permeability and the

effective therapeutic protein available for permeation and its concentration in the GIT fluid. Moreover, is further dependent on the compound's specific physicochemical proprieties as lipophilicity, molecular weight, size, and surface charge that influence the pharmacokinetics and biodistribution of the protein.

1.1.2. Strategies do overcome therapeutic proteins challenges

There are several approaches to overcome the therapeutic protein's challenges, that target one or more of the problems mentioned in section 1.1.1. These strategies can be coupled into four categories: amino acid manipulation, post-translation modification, bioconjugation, and carrier-mediated delivery.

The amino acid manipulation techniques consist of inserting, deleting, or altering one or more amino acids in the proteins chain, which has been proven to reduce immunogenicity and proteolytic cleavage *in vivo*.

Taking into account that the immunoglobulin G has a long circulating serum half-life (~3 weeks) through pH-dependent FcRn binding-mediated recycling, a study was conducted to extend serum persistence of non-antibody therapeutic proteins, by taking advantage of the intracellular trafficking and recycling mechanism of IgG. The results showed an improvement in the serum half-life of engineered FcγRIIa fusion, which suggest that this strategy has the potential to prolong the half-life of therapeutic proteins (46).

One of the strategies employed to reduce renal clearance rate is increasing protein size and molecular weight. Therefore, post-translation modification consists of attaching the protein to polymers that can be either natural or synthetic to increase their hydrodynamic volume, prevent rapid renal clearance and thereby increase the protein serum half-life (47). The proteins conjugates with more clinical and commercial success have been with polyethylene glycol (PEG), a non-toxic and non-immunogenic polymer approved for internal use - Figure 1.5 (A). PEG's main advantages are its solubility in both aqueous and organic milieu, presenting great flexibility, high hydration that consequently increases its hydrodynamic volume, and a range of molecular weight species gallowing tunable properties. All these proprieties are also acquired by the therapeutic proteins bonded covalently to PEG, in a process called PEGylation. The water cloud surrounding the protein conjugated with PEG will favour increase solubility,

become resistant to antibodies, proteolytic enzymes and cells and, due to their increased size, are more slowly filtered by the kidneys (48).

The main foundation of bioconjugation approaches is that during hepatic metabolism, proteins are taken by hepatocytes generally, receptor mediated. After that, they are degraded in the lysosome by enzymes and cleared out of circulation. However, some endogenous proteins can avoid liver metabolism, by imitating the specific receptor-mediated recycling of endogenous proteins. Therefore, it was observed that binding of therapeutic proteins to some endogenous proteins, as albumin or immunoglobulin, receptor coupled recycling helps target protein recycle back to circulation as their moieties and, therefore, it can be used as a strategy to avoid enzymatic degradation, extending the half-life of therapeutic proteins – Figure 1.5 (B) (47).

Carriers are used with the purpose of protecting the protein against the *in vivo* environment. Apart from protection, some carriers also can enable targeted and controlled release of the protein (47). Nanoparticles as carrier systems will be the focus of this work and therefore this topic is further deepened in section 1.2.

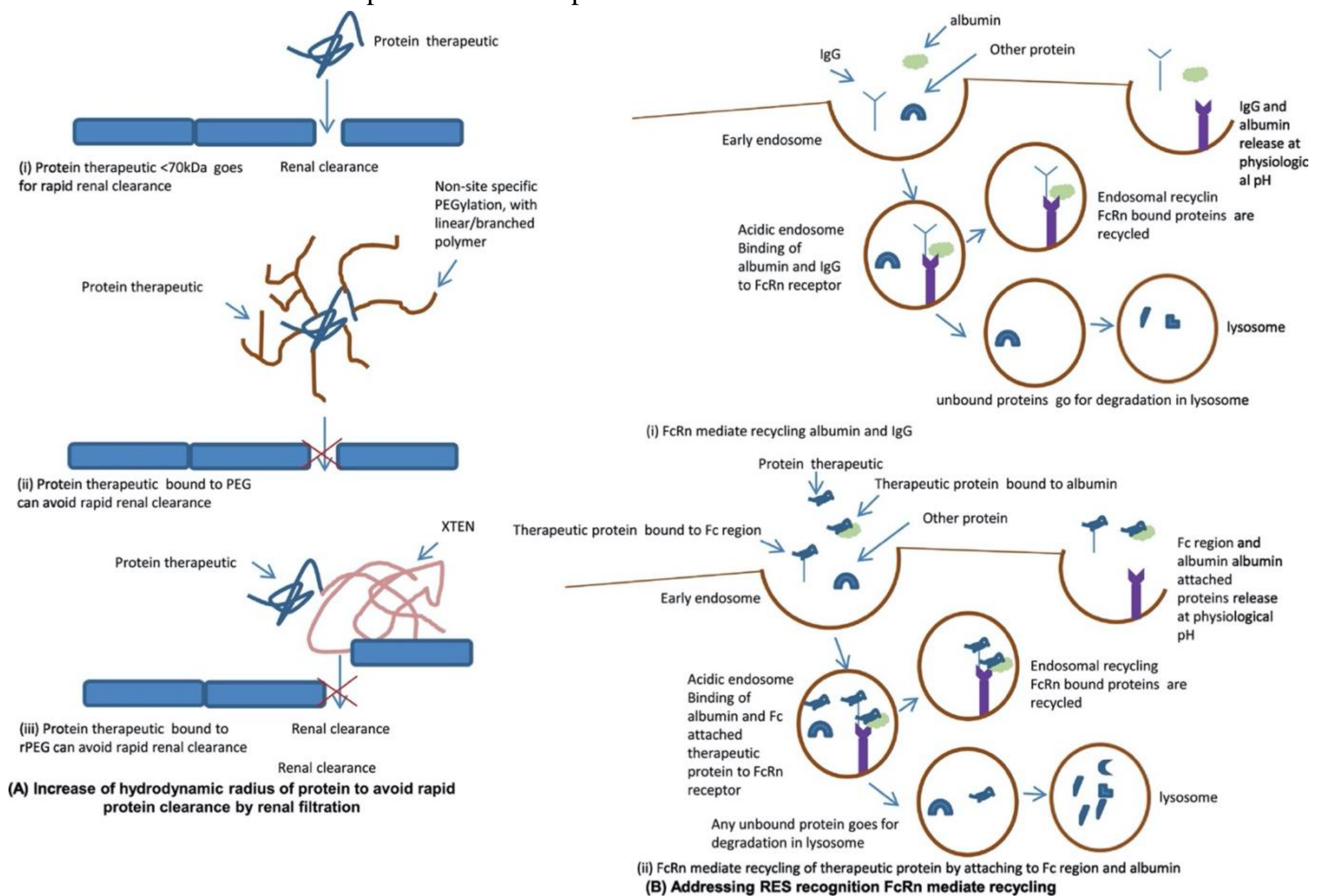

Figure 1.5. Receptor-mediated recycling (A) and protein half-life extension strategies: avoidance of rapid renal clearance (B). *Adapted from* (19)

1.2. Nanocarriers as tools to improve therapeutic proteins delivery

The development of nanotechnology represents one of the most revolutionary and promising technologies of the XX century. Nanoscience is the study of structures and molecules on the nanometer scale and nanotechnology is its practical application (49) (50). Nanotechnology is the manipulation and control of matter on the nanoscale dimension, which ranges from 1 to 1000 nm, applied to several industries and in biomedical scientific knowledge (51). In Figure 1.6 it is represented the nanoscale and a comparison with other materials. The prefix "nano" derives from the Greek word that means "dwarf" or reduction in size, corresponding to a one thousand millionth of a meter reduction. This reduction, along with the ability to control and manipulate structures in nanoscale enables the exploration of new physical, biological, and chemical properties of systems (52).

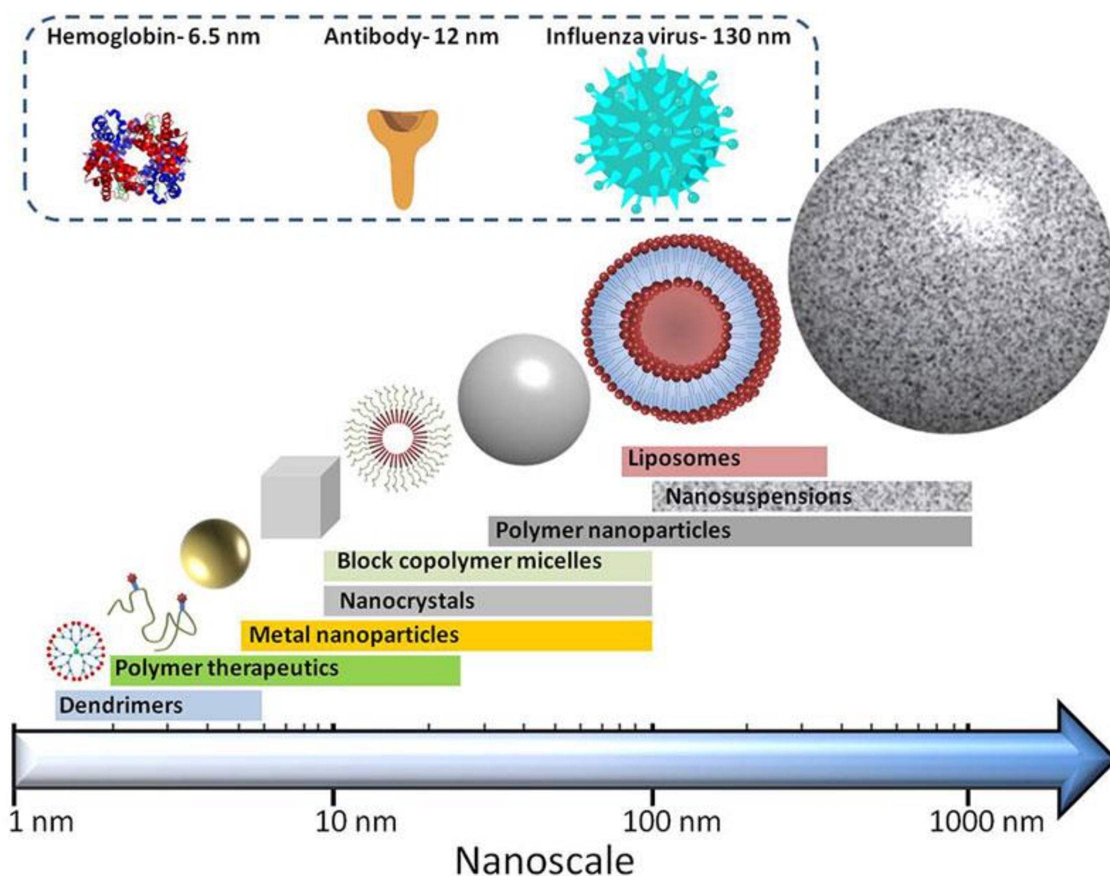

Figure 1.5. Nanoscale representation and size comparison with other structures. *Reprinted with permission from* (53)

In 1959 the physicist and Nobel Prize winner Richard Feynman first introduced the concept of nanotechnology when he presented a lecture entitled "There's Plenty of Room at the Bottom" at the California Institute of Technology, proposing the hypothesis "Why can't we write the entire 24 volumes of the Encyclopaedia Britannica on the head of a pin?" to explain his vision of using machines to construct smaller machines, down to the molecular level (54). Ever since a great advance has been made and nanotechnology is now applied in several areas as physics, chemistry, computer science and biology. Several studies proved the huge potential of nanotechnologies in biomedicine for the diagnosis and treatment of several diseases, with significant advances in this field, especially for cancer treatment due to the potential to overcome the limitations of the traditional approaches (55).

Drug discovery is a time-consuming, arduous, expensive, and high-risk process, with a significant low success rate and several challenges to overcome. Furthermore, in the last decades, it became evident that drug development alone is not sufficient to secure progress in drug therapy. The main reasons for therapy failure include insufficient drug concentration due to pharmacokinetics proprieties and inconstant plasma levels because of the pharmacodynamic influence. It is also due to the lack of specificity of some drugs and poor drug solubility. Recognizing these aspects, the development of suitable drug carrier systems emerged as a promising solution (56,57).

Drug delivery systems are used to enable controllable drug release and improve both the safety and efficacy of the drugs. Nanotechnology has begun to be implemented for this purpose and other than satisfying the mentioned goal of drug delivery systems, also targets the loaded drugs into specific body locations. Consequently, the main objectives of nano-bio technologies include more specific drug targeting and delivery, reduce toxicity while maintaining therapeutic effects, enhance safety and biocompatibility and accelerating the new medicines development process. Even though drug delivery systems do not modify the pharmacokinetic or pharmacodynamic of the drug, they can modulate it, enabling long-acting therapeutic formulations. The mentioned modulation is based on the concept of incorporating the protein into a matrix or into another molecule that will work like a "protective covering". This cover will also function as a depot that instead of releasing all the therapeutic at once, will gradually release it in circulation, creating a long-acting formulation (19,58).

Nanoparticles are very attractive as drug delivery systems due to their unique characteristics as the surface to mass ratio considerable higher when compared with other

particles, ability to adsorb and carry other compounds such as drugs and proteins and enhanced solubility and diffusivity. All these characteristics of nanoscale materials, in particular, the enhanced solubility and diffusivity have been proven to increase the blood circulation half-life (59). Typically, the size of the nanoparticles ranges from 1 to 1000 nm. However, for nanomedicine purposes, sizes smaller than 200 nm are preferable due to the ability to traverse micro-capillaries. On the other hand, particle sizes above 100 nm may be required for loading enough drug (58,60). Apart from the advantages, there are also significant disadvantages to the use of nanoparticles. Burst release of the therapeutic and the consequent side effects, poor loading efficiency and manufacturing and administration challenges are some of the most frequent (19,61).

There are several classifications of nanoparticles according to their morphology, size and chemical properties. Considering the composition materials of the nanoparticles they can be divided into 4 categories:

1. Carbon-based nanoparticles – Fullerenes and carbon nanotubes (CNTs) are the biggest subclasses. Fullerenes are composed of a globular hollow cage form of pentagonal and hexagonal carbon units, especially interesting due to their electrical conductivity, high strength, structure, electron affinity and versatility. CNTs are elongated tubular structures, structurally similar to a rolling graphite sheet. These are frequently used for commercial applications fillers and efficient gas adsorbents for environmental purposes (62–64).
2. Metallic nanoparticles – Made by metals precursors with unique optoelectrical properties which make them very valuable for applications in research areas (63).
3. Polymeric nanoparticles – Usually organic-based NPs, in their majority with nanosphere or nanocapsules shape with a big range of applications (65,66).
4. Lipid-based nanoparticles – Made of lipids both in solid or liquid state. It is fully addressed in the next section.

1.3. Lipid nanoparticles as carriers for therapeutic proteins delivery

The use of lipid nanoparticles as drug carriers have been studied for many years, including oil-in-water (O/W) emulsions, liposomes, microparticles and nanoparticles. The first emulsion introduced to clinical as carrier systems, in the fifties, was only intended to reduce the drug side effects. Although accomplishing the intended goal, they did not had the expected success, which can be explained by the physical instability that can be caused by the incorporated drug and the low solubility of the used lipids. Later, in 1965, liposomes were developed by Bangham *et al.* and introduced as drug delivery systems in 1986, by Dior® in the cosmetic market. A few years later, at the end of the eighties/beginning of the nineties, liposomes started being used in pharmaceutical field as drug delivery systems. Even so, and same as for the O/A emulsions, the number of products on the market is still limited, in part due to the high cost of pharmaceutical liposomes (67).

The therapeutic effectiveness of therapeutic proteins depends on its bioavailability which can be defined by the ability of a compound to reach the site of action at a rate and amount necessary to illicit the therapeutic effect. For most of the drugs, the therapeutic effect is directly related to the plasma levels which means that the term bioavailability can be defined as the rate and extent of absorption of unchanged drugs from its dosage form (68,69). The use of lipid nanoparticles has many advantages including improvement of bioavailability. Furthermore, the lipid nanoparticles advantages compared to other particulate systems include (69,70):

- Biocompatible and biodegradable lipids
- Low toxicity
- Targeted and control drug release
- Encapsulation of both hydrophilic and hydrophobic compounds
- Ease scalability of production methods

Therapeutic proteins are highly vulnerable molecules due to their physical and chemical instability. They are often administered intravenously to overcome their short-half life. Furthermore, they also present a poor capacity of penetrating membranes, which is a considerable limitation for their administration by other administration routes. The use of lipid nanoparticles as drug delivery systems allows to overcome several limitations of therapeutic proteins. In one hand, the lipid nanoparticles structure protects the

therapeutic protein structure from degradation, but it also increases their bioavailability and capacity to penetrate membranes. The lipid nanoparticles allow the entrapment of both lipophilic and hydrophilic compounds as proteins, and fulfil the requirements to be used as an optimal drug delivery system. The encapsulation of therapeutic proteins into lipid nanoparticles can address the major limitations of the therapeutic proteins and open a completely new window of opportunities for their application and routes of administration (8).

The main features of SLN and NLC are represented in Figure 1.7, and further details on both types of lipid nanoparticles will be discussed in the following sections.

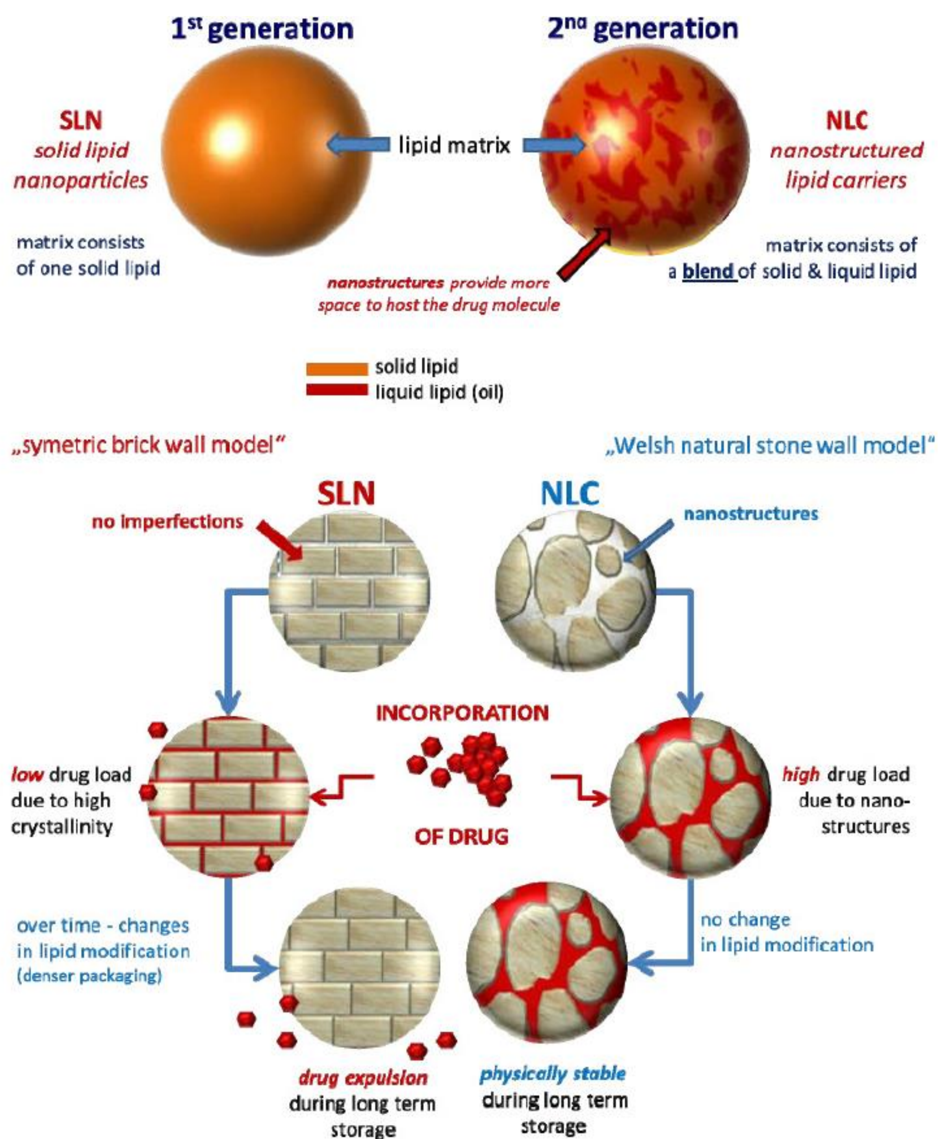

Figure 1.6. Differences from the SLN and NLC matrix's structure. *Reprinted with permission from* (71)

Table 1.3. Frequently used excipients to produce SLN and NLC. *Adapted from* (72)

Ingredients	Examples	Properties
Solid lipids	Beeswax	Natural wax with GRAS status and MP of 62-64°C; requires HLB of 9
	Carnauba	Natural wax with GRAS status, MP of 82-85°C; requires HLB of 12
	Cetyl palmitate	Synthetic wax with MP of 40.5-51°C; requires HLB of 10
	Compritol® 888 ATO	Blend of esters of behenic acid with glycerol; MP of 69-74°C Acceptable safety profile and established as emulsifier, with HLB of ≈2
	Dynasan®	Series of natural and safe triglycerides with different MPs
	Gelucire®	Series of lipid defined by their MP between 33-70°C and HLB between 1-18 Gelurice 50/13 is GRAS listed and the most frequently used for SLN/NLC
	Precirol® ATO 5	Glyceryl palmitostearate, mixture of mono, di and triglycerides of palmitic and stearic acid, with GRAS status, MP of 58°C and HLB of 2
	Softisan® 378	Blend of triglycerides with hydrocarbon with GRAS status and MP of 35-42°C
	Stearic acid	Endogenous fatty acid with GRAS status, MP of 70°C and HLB of ≈15
Liquid lipids	Miglyol® 812	Triglycerides of capric and caprylic acid with GRAS status, high stability against oxidation and high solubility for many drugs
	Oleic acid	Pure substance used as emulsifying agent and penetration enhancer with GRAS status
	Squalene	Triterpene produced by human skin cells (as precursor for cholesterol)
	Vitamin E/alfa-tocopherol	Offers sensitive substances protection against oxidation
Surfactants	Lecithin	Component of cell membranes, used in a wide variety of pharmaceutical applications as emollient, emulsifying and solubilizing agent, with HLB between 4–9
	Plantacare® 810	Caprylyl/capryl glucoside, high effective stabilizer for SLN and NLC with HLB of 15-16
	Poloxamer® 188	Used as emulsifier and stabilizing agent in a wide variety of pharmaceutical formulations, it is nontoxic and nonirritant, with HLB > 24.
	Quillaja saponin	Natural saponin-based surfactant with antioxidant properties and HLB of 13.5
	Sodium lauryl sulfate	Anionic surfactant, widely used in cosmetics and pharmaceutical formulations, moderately toxic but with GRAS status and HLB ≈ 40
	Tween® 80	Polysorbate 80, an O/W surfactant with GRAS status widely used and HLB of ≈15

1.3.1. Solid Lipid Nanoparticles

SLN were developed by Müller *et al.* in 1993 and in parallel by Gasco *et al.* (73,74). SLN brought attention due to its advantages, being able to assemble the advantages of other colloidal carriers while avoiding some of their disadvantages. These nanoparticles are interesting delivery systems that have shown great advantages. These include:

- Allowing control drug release and targeting
- Increasing drug stability and safety
- Allowing high drug payload
- Incorporation of lipophilic and hydrophilic drugs
- Use of Generally Recognized as Safe (GRAS) compounds and therefore low toxicity as carriers
- Avoidance of organic solvents
- Large-scale production methods for their production

They have been actively investigated for the delivery of drugs by different administration routes (57,72,75). The general formulation of SLN includes solid lipid(s), emulsifier(s) and water, and the most frequently used ingredients for SLN production are shown in Table 1.3.

The lipid(s) composes the matrix of the SLN, and these are solid at both room and body temperature, usually with a melting point above 40ºC, used in a concentration ranging from 5 to 40%. There is a wide range of lipids used, from triglycerides, partial glycerides and fatty acids to steroids and waxes, being all biocompatible/physiological and biodegradable lipids, which represents one of the greatest advantages of the SLN because it decreases the danger of acute and chronic toxicity. The choice of lipid will depend on the solubility of the compound that will be incorporated.

The emulsifier role in the formulation is to reduce the tension between the aqueous and lipid phases, thereby helping the stabilization of the system. Since they are amphiphilic molecules, they are usually placed in the interface of the system. Different types of emulsifiers have been employed in SLN formulations, and it was discovered that a binary combination of emulsifiers helps to stabilize the systems more effectively. The

choice of emulsifiers will take into consideration the hydrophilic-lipophilic balance (HLB) of the lipids employed in the formulation, well as their concentration in the lipid phase and the administration route (57,72). The most frequently used ingredients in SLN formulations are shown in Table 1.3.

There are three incorporation models for the SLN that differ on the location and distribution of the loaded therapeutic protein within the lipid core as shown in Figure 1.8.

- SLN Type I or homogeneous matrix model – In this model, the drug is molecularly dispersed in the lipid core or as amorphous clusters. Usually, it is obtained when the production method is the high-pressure homogenization (HPH), either cold HPH or hot HPH in an optimized drug/lipid ratio. This type allows to produce nanoparticles with good controlled release proprieties.

- SLN Type II or drug enriched shell model - At this model a drug-free or drug reduced lipid core is obtained, contrasting with an outer shell containing the drug and lipid. This model can be used when the intention is not to obtain a prolonged drug released but a burst release of the encapsulated drug.

- SLN Type III or drug enriched core model – This model consists of a drug enriched core with a lipid coverage. It is obtained when the drug concentration in the lipid melt is closed, or at saturation solubility. Under cooling, the saturation solubility is exceeded and the drug precipitates. This model is also suited for prolonged drug release (70,76).

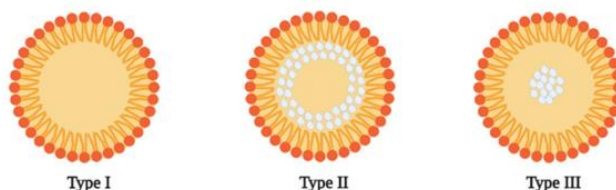

Figure 1.7. Types of SLN. Adapted from (76)

Nevertheless, SLN have two main limitations related to their crystal structure: low loading capacity and drug expulsion during storage. Drugs are mostly incorporated

between the fatty acid chains, between lipid layers or in the amorphous clusters of the crystal imperfections. SLN usually crystalize in a perfect lattice, specially those obtained by highly purified lipid, which explains the low encapsulation efficiency, since the more densely packed the crystal is the less drug is possible to incorporate(77). Furthermore, during storage, the lipid molecules suffer a time-dependent restructuration process in which the more perfect lipid crystalline structures lead to the expulsion of the drug (78).

In a study where BSA was used as a model protein for the encapsulation into a matrix modified by incorporation of lecithin into the lipid matrix or different emulsifier concentrations, the obtained particle payload with BSA was between 2,5 and 15% and seemed to be commanded by the particle surface characteristics, particularly the surface charge and the specific surface area (79). In a different study, using SLN for the encapsulation of lysozyme, the method produced formulations with reduced concentration of protein and low encapsulation efficiency, which considering the high costs of some therapeutic proteins and the waste generated by a reduced encapsulation efficiency, represents a limiting factor and urges the need of developing of further improved lipid nanoparticle formulations (80).

1.3.2. Nanostructured lipid carriers

NLC were developed to overcome the main limitations of the SLN that could compromise the applicability of the formulation: the low drug loading capacity and drug expulsion during storage. Therefore, it was investigated possibilities to improve the SLN formulation, being discovered that adding a liquid lipid into the solid matrix of the SLN increases the imperfections on the matrix, which leads to a higher loading capacity while maintaining the stability of the formulation. NLC are composed of an unstructured solid matrix composed of a mixture of solid and liquid lipid, and an aqueous phase containing one or more surfactants. In general, the lipids are mixed in a 70:30 up to 99.9:0.1 solid/liquid ratio and the concentration of the surfactant(s) ranges from 1.5% to 5% (w/v). The ingredients employed in the production of NLC are the ones used for SLN plus a liquid lipid, all of them marketed products or approved by regulatory agencies – Table 1.3.

Same as the SLN, according to the production method, and the composition of the lipid mixture, there are three types of NLC – Figure 1.9.

- **The imperfect type** – Occur when spatially different lipids are mixed, composed of fatty acids that introduce imperfections in the crystal matrix. These imperfections allow a higher drug loading capacity, which can be further increased by using different glycerides and varying the saturation and length of the carbon chain.

- **The amorphous type** – At this model, it is used solid special lipids as hydroxyoctacosanyl hydroxy stearate or iso-propyl myristate with a liquid lipid, forming a structureless amorphous matrix. The resulting amorphous state instead of an ordered state avoids β-modification during storage and therefore the drug expulsion.

- **Multiple oil-in-solid fat-in-water (O/F/W) type** – This last model results in numerous nanosized liquid oil compartments disseminated in the solid matrix. In this case, the drug solubility is higher in the oil compartments, which increases the loading capacity and the prolonged release because the compartments are surrounded by solid lipid (70,76).

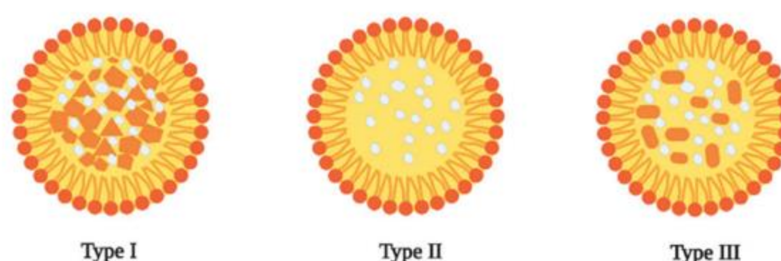

Figure 1.8. Types of NLC. Adapted from (76)

In the last years, the number of research on NLC formulations increased considerably and it has been emerging as an ideal drug delivery system for the pharmaceutical market. The advantages of these nanoparticles include the ones mentioned for the SLN well as high loading capacity and stability over storage.

In a recent study, it was developed coenzyme Q10-loaded (co-Q10) NLCs by the high shear homogenization method, succeeded to obtain spherical loaded nanoparticles with an average particle size of 180-350 nm, PDI below 0,5, zeta potential below -0,3 mV and an encapsulation efficiency between 83 to 88% (81). Nevertheless, there is no description

in the literature of therapeutic proteins encapsulated into NLC or produced by methods that would not damage the protein tridimensional structure.

In the literature there is no description of NLC for the encapsulation of therapeutic proteins. Furthermore, all the production methods described employ temperature and pressure, which is not suited for the encapsulation of therapeutic proteins. From the several methods available to produce NLC, the HPH and microemulsion technique are the preferred methods (77,82). Since NLC are produced by emulsification, it is necessary to have both the lipid and the aqueous phases in the same physic state, which can be obtained either by melting the lipid or dissolving it in an organic solvent. Avoidance of organic solvents is preferable, but for therapeutic proteins the employment of temperature is not the best option because it can damage the protein structure.

With the emerging importance of therapeutic proteins, and all the advantages previously mentioned, their encapsulation into NLC using a method that do not damage the protein structure, can possible change the paradigm of therapeutic proteins, allowing their administration by different administration routes and optimize their use as therapeutics.

1.4. Production methods of SLN and NLC for therapeutic proteins delivery

Both SLN and NLC are produced using the same methods, which are shown in Figure 1.11 and are further detailed in this section (72,83). From the several methods used to produce SLN and NLC, the choice of the most suitable relies on the therapeutic protein to be encapsulated, the type of lipids, and the administration route. It is important to notice that not all the methods can be used for the encapsulation of therapeutic proteins, since it is important to use methods that do not damage the protein structure, which means that stress conditions as temperature and high pressure should be avoided. All the forward described methods are well established to produce SLN and NLC. Nevertheless, they are not optimized for the encapsulation of therapeutic proteins, which are shear and temperature-sensitive compounds, and therefore require methods that avoid thermal and mechanical stress, to protect the therapeutic protein structure(79).

Therefore, in the next sections the most frequently used production methods to produce lipid nanoparticles are described.

1.4.1. High-pressure homogenization (HPH)

High-pressure homogenization emerged as a reliable, well established, and widely used technique for the production of lipid nanoparticles. Some of the advantages of this method include the possibility of large-scale production, avoidance of organic solvents and obtained particles with an average size on the submicron region. For this technique it is used homogenizers that push the liquid with high pressure, between 100-2000 bar, through a very narrow gap in the micron range, making a high acceleration of the fluid (over 1000 km/h) in a short distance. The shear stress and cavitation forces inherent to the process will reduce the particles size into the submicron range. It is important to notice that the high pressure involved leads to an increase in temperature, which is a limitation of the method, and needs to be addressed when therapeutic proteins are intended to be loaded into lipid nanoparticles.

There are two variations of this technique, the hot and cold HPH, performed respectively at high and low temperature – Figure 1.10. Even so, both techniques, require a heat evolving preparatory step, which is the dissolution or dispersion of the drug in the lipid melt, using temperatures at least 5º C above the lipid melting point (57,72). The hot HPH is the less adequate for the encapsulation of therapeutic proteins due to the high temperatures applied during the emulsification process. The cold HPH can be considered as an option for the encapsulation of therapeutic proteins if the pressure applied is controlled to not damage the therapeutic protein.

Lysozyme was used as a model protein for optimizing the incorporation of therapeutic proteins into SLN using both variations of the HPH method. The results showed that the protein remained intact during all the harsh conditions of the procedure, but the encapsulation efficiency was only about 59% and the protein tended to partition to the aqueous phase. These results were not completely surprising because the lysosome is a protein with high structural stability (8,80). On the other hand, results using bovine serum albumin (BSA) showed that the temperature and pressure conditions of HPH strongly affected the protein structure. Other studies using human insulin and cyclosporine A showed that both proteins maintained their structures, with cyclosporine A showing incorporation efficiencies above 90% (8).

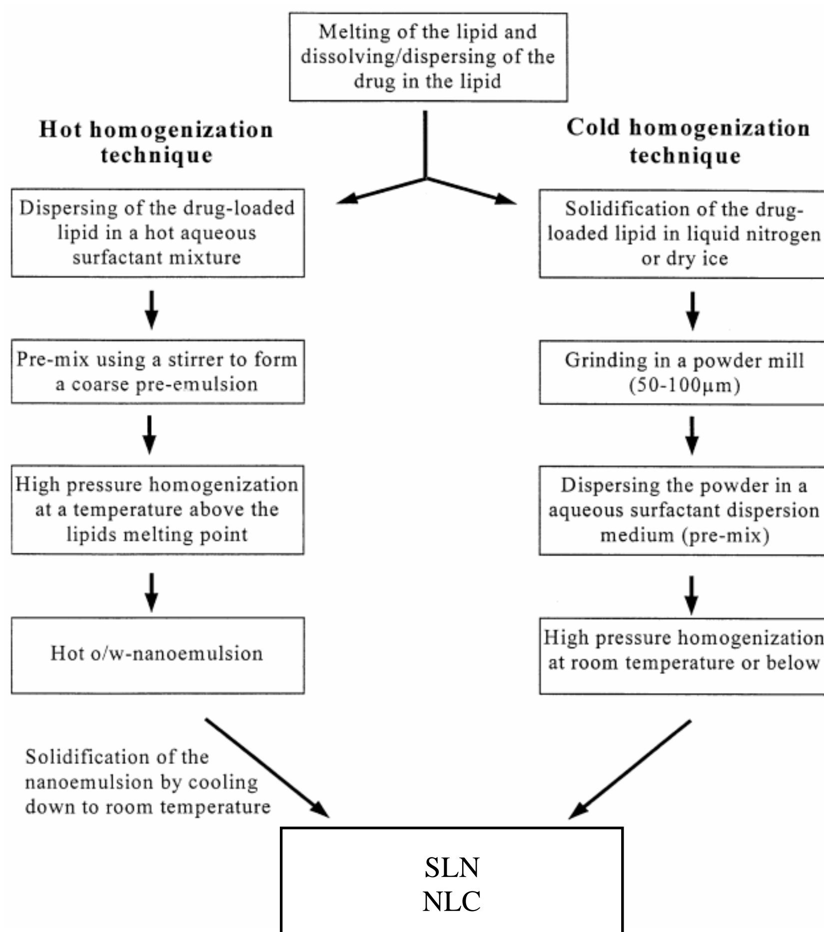

Figure 1.9. Schematic representation of Hot and Cold Homogenization Techniques (58)

1.4.1.4. Hot High Pressure Homogenization

In this technique, the entire process is performed at temperatures above the lipid melting point. First, a pre-emulsion is prepared, with the drug-loaded lipid melt and the aqueous emulsifier, both at the same temperature, under high shear stirring. After that, the formed pre-emulsion is homogenized by HPH. High temperatures frequently lead to lower particle size but, on the other hand, they can also increase the degradation rate of the system. Usually, one cycle of homogenization is sufficient to produce SLN and NLC with an average particle size ranging from 250 to 300 nm, when the pre-emulsion concentration is between 5-10%. When the concentration is higher than 30% is no longer possible to produce NLC, but highly concentrated SLN can still be obtained. In these cases, it is important to adjust the number of homogenization cycles since the energy required to shear the lipid mass is proportional to its concentration in the formulation. However, it is important to notice that increasing the number of homogenization cycles

also frequently leads to bigger particle size, because increased particle kinetic energy, favors coalescence. At this point, it is obtained an emulsion due to the physic state of the lipid. The final step is cooling the sample at room temperature or lower, leading to lipid crystallization and formation of the solid nanoparticles.

According to the literature, this technique can be used for some heat-sensitive-compounds because the time of exposure to high temperatures is relatively short. Even so, the temperature employed is one of the limitations of this technique since, as mentioned, it won't be suitable for highly sensitive compounds and hydrophilic compounds that in high temperatures can partition from the lipid phase to the aqueous phase (57,72). Nevertheless, there is no relevant description in the literature of therapeutic proteins encapsulated into lipid nanoparticles using this production method. The reason for this is because therapeutic proteins are highly temperature sensitive compounds, and therefore the use of temperature would damage the tridimensional structure of the proteins and ultimately compromise its therapeutic effect.

1.4.1.5. Cold High Pressure Homogenization

This technique was developed to overcome the limitation of hot homogenization: degradation of the loaded bioactive due to high temperature exposure, drug partition into the aqueous phase and the complex crystallization step being recommended for extremely heat sensitive and hydrophilic compounds, by reducing the temperature exposure but not preventing it completely. After the preparatory step, the obtained mixture is rapidly cooled down to a solid state, using dry ice or liquid nitrogen, favoring a homogenous distribution of the bioactive. Then, the solid obtained is turned into microparticles. First, a pre-suspension is prepared by dispersing the obtained microparticles in a cold emulsifier solution and then, the mixture is subjected to HPH, at or below room temperature, forming the lipid nanoparticles. For this method, five cycles at 500 bars are usually performed to obtain SLN and NLC.

The big disadvantage of this technique is the need to employ high energy during the homogenization step. Also, the particles formed are usually bigger and more polydisperse than the ones formed using the hot HPH (57,72).

1.4.2. Emulsification methods

The emulsification methods are the best ones to load therapeutic proteins into lipid nanoparticles, mainly due to the avoidance of high temperature and shearing stress. In this section several emulsification methods are address.

1.4.2.4. Ultrasonication

Ultrasonication is a dispersing technique, on which the lipid nanoparticles are obtained by dispersing the melted lipid in the aqueous phase with the surfactant. It allows the cleavage of large particles into smaller ones, by providing sound energy, usually above 20 kHz of ultrasonic rates/frequencies for homogenization (70,84). In the first step of this technique, the lipid is melted, approximately 5-10 ºC above its melting point. Then, the melt is dispersed in an aqueous surfactant, at the same temperature, under high stirring to form an O/A emulsion. The formed emulsion is subjected to sonication to reduce the droplet size. In the final step, the emulsion is cooled at a temperature under the solidification temperature of the lipid, with the formation of a nanoparticle dispersion (70). Some of the advantages of this technique relate to the equipment used, which are common laboratories material. However, the energy distribution during sonication is not homogenous, resulting in highly polydisperse particles (72).

To evaluate the influence of sonication time and pulse frequency on average dispersion, temperature, particle size and zeta potential, SLN were prepared using a 1:3 ratio of stearyl alcohol (SA) and cetyltrimethylammonium bromide as lipids, applying different sonication time and pulse frequencies, respectively 5, 10 and 15 min and 30, 60 and 90%. The values were selected based on the results from a preliminary study. During the sonication process, only the pulse frequency and sonication time were varied, maintaining all the other parameters constant. The desired SLN size was 100 nm, which was obtained with 60% pulse frequency at 40% power for 10 min. These optimized sonication parameters were used to study the influence of the lipid choice on size and zeta potential, applying the same parameters using different lipids. The resulting SLN were after tested to determine the short-term stability in aqueous dispersions. The mean particle sizes of SLNs made with SA, cetyl palmitate, Precirol, Dynasan118 and Compritol were 98, 190, 350, 350 and 280 nm, respectively. The obtained results suggested that an increase in pulse frequency and sonication time produces smaller nanoparticles, unwanted increase in dispersion temperature but an irrelevant influence on zeta potential. It was

also found that increasing the length of the hydrocarbon tail of the lipids increases the size of the nanoparticles (85).

1.4.2.5. Double emulsion technique

This approach consists of emulsifying a heated aqueous solution of the drug in the previously melted lipid, forming water in oil (w/o) emulsion, stabilized with proper excipients. Then, the formed w/o emulsion is dispersed in an aqueous solution of a hydrophilic emulsifier, forming a double water-in-oil-in-water (w/o/a) emulsion. Finally, the emulsion is cooled under stirring, forming the solid lipid nanoparticles. This technique is suitable for the incorporation of hydrophilic therapeutic proteins but, on the other hand, the obtained particles are relatively large (70). In a study using cetyl palmitate, glyceryl tripalmitate and glyceryl palmitostearate as the lipids for the preparation of SLN, using the double emulsion technique, the nanoparticles were successfully prepared and their size were 447.5 ± 50.8, 444.8 ± 72.5, and 213.7 38.4 nm, respectively (86).

In another study using thymopentin and insulin as the model protein drugs, it was prepared a novel Gel-Core-solid SLN using a double emulsion technique. The goal of this work was to enhance the entrapment efficiency. It was favourably obtained the Gel-Core-SLN with a particle size of 305.2 nm and zeta potential of -17.15 mV. The entrapment efficiency, the parameter under analysis, of thymopentin-loaded Gel-Core-SLN and insulin-loaded Gel-Core-SLN were 61.97% and 57.36%, respectively, with both presenting low burst release. In terms of pharmacological availability of insulin-loaded Gel-Core-SLN the value was 6.02%. Therefore, this study showed promising results for the Gel-Core-SLN as a drug delivery system prepared by a double emulsion technique (87).

1.4.2.6. Microemulsion technique

The first step of this homogenization technique consists on placing both phases at the same temperature, by melting the lipid or blend of lipids and heating the aqueous phase containing the surfactant. Once both phases are at the same temperature, the aqueous solution is added to the lipid solution, under mild stirring, to create the microemulsion. Then, to obtain the nanoemulsion the system is dispersed in cold water with a temperature ranging from 2 to 10ºC, under mild mechanical mixing, thus ensuring

that the reduced particle size is due to the precipitation and not because of the mechanical stirring process. The last steps are washing the system using distilled water, filtering it to remove the larger particles and finally lyophilizing the system to remove the excess water (88). The big advantage of this technique is allowing the preparation of the particles under mild temperature and pressure conditions. Some of its disadvantages are the need for a high concentration of surfactant, the dilution of the system and therefore obtention of a relatively dilute system, with low particle concentration (7,72).

The first attempt to encapsulate peptide drugs in SLN was carried by Morel et al. using this technique for the encapsulation of triptorelin and hymopentin as model peptides (89,90). The encapsulation efficiency was low in both cases and similar results were observed for the encapsulation of cyclosporine A (91).

1.4.2.7. Solvent emulsification/ Solvent evaporation

In this method, a nanoparticle dispersion is obtained by precipitation of o/w emulsions. First, the lipophilic compounds are dissolved in an organic water-immiscible solvent. The obtained mixture is then emulsified in an aqueous phase, forming an o/w emulsion. The organic solvent is then evaporated, under reduced pressure, leading to the precipitation of the lipid in the aqueous medium and subsequent formation of a nanoparticle dispersion (57,92). Other than the advantages mentioned for the solvent injection, this approach also avoids temperature and high-energy sources, and it results in particles with a narrow size distribution (72,93).

Overall, this is a widespread method in the preparation of nanoparticles, including SLN. For hydrophilic compounds, including proteins and antigens, associating the double emulsion technique (w/o/w) to this method demonstrated to improve their encapsulation efficiency. For that reason, a big part of the studies with protein antigen encapsulation in solid lipid nanoparticles is based on this method because it also avoids the use of temperature or pressure conditions. However, the use of organic solvents can increase the toxicity of the final product.

This method was used for the encapsulation of insulin, resulting in a 45% burst release. The same authors using calcitonin as model protein and different materials were able to demonstrate the feasibility of the method, obtaining encapsulation efficiencies above 90%. In a study conducted to improve the oral absorption of insulin, an insulin-loaded Vitamin B12 (VB12)- gel core solid lipid nanopaticles (GCSLN) was prepared by

a combination of double emulsion and solvent-evaporation methods. The results of this study were very promising for the use of VB12-GCSLN containing insulin as carrier for drug delivery. The VB12-GCSLN had an encapsulation efficiency (EE) of 55.9%, a burst release of less than 10% in the first 2 h, an absorption of insulin with a relative pharmacological availability of 9.31% and considerable stable blood glucose levels up to 12h (94).

1.4.3. Solvent injection

In this method it is prepared a transitional o/w emulsion using a partially water-soluble solvent that is firstly saturated in water, to guarantee initial thermodynamic equilibrium. The fundament of the technique is the partial solubility of the compounds in water. Firstly, the lipids are dissolved in a water-miscible solvent forming a mixture rapidly injected by an injection needle, into an aqueous surfactant solution under continuous stirring, causing the organic solvent to diffuse into the water, leading to droplet size decrease and consequent formation of the nanoparticles (70). This method uses mild organic solvents, avoids several critical as high temperatures, high pressures and high emulsifier concentrations, and has emerged as an efficient, versatile, and easy to implement technique (72,95).

The solvent injection method was firstly used for the production of SLN and NLC in 2003 by Schubert et al. The results from that study showed that acetone, ethanol, isopropanol and methanol are suitable solvents for the preparation of lipid nanoparticles, which was not verified with ethylacetate that was not able to successfully produce the nanoparticles. The particle sizes obtained were between 80 and 300 nm depending on the preparation conditions. It was also performed a physicochemical characterization of the particles that revealed a decrease in crystallinity of the colloidal lipid in comparison to the bulk lipid (96).

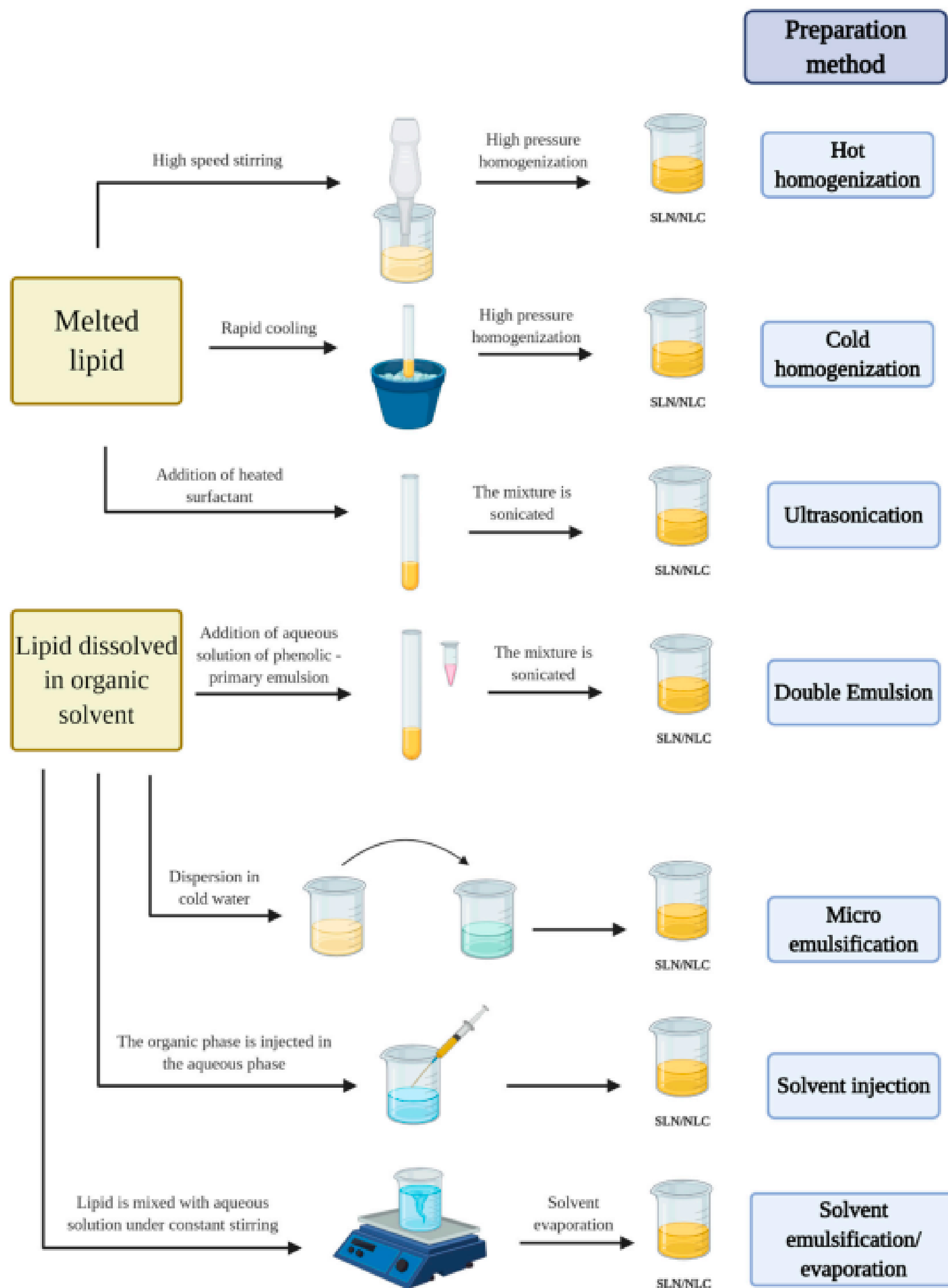

Figure 1.10. Methods used for SLN and NLC preparation. Adapted from (77)

2. Objectives

The SLN have been studied for the encapsulation of therapeutic proteins but unfortunately, their low loading capacity and expulsion of the encapsulated compound during storage limits their use for the encapsulation of therapeutic proteins. According to the literature, it is expected that NLC will avoid these two problems. Nevertheless, the described methods for the encapsulations of compounds into NLC present in the literature cannot be applied to therapeutic proteins because they use stress factors as high temperature and/or pressure.

The main goal of this work was to develop an optimized production method suitable for the encapsulation of therapeutic proteins into NLC, to secure the preservation of the protein structure, avoiding temperature and other stress conditions like pressure, that can interfere with their structural stability and bioactivity. Successfully encapsulating therapeutic proteins into NLC would ultimately allow their administration by different administration routes.

Specifically, the objectives of this work are:

1. Perform an overview about the application of lipid nanoparticles for therapeutic proteins delivery
2. Develop an optimized production method for the loading of therapeutic proteins into NLC, without using heating or other methodology that could degrade therapeutic proteins
3. Characterize the administration system developed in terms of size, diameter and polydispersity index (PdI), zeta potential, association efficiency and loading capacity
4. Compare the ability of the NLC with the SLN used as control, for the administration of therapeutic proteins

3. Materials and methods

3.1. Materials

The solid lipid used for lipid nanoparticles production was Suppocire from Gattefossé SAS (Saint-Priest, France), whereas the liquid lipids were Oleic Acid from Sigma–Aldrich Chemie Gmbh (Steinheim, Germany), and Capryol and Miglyol from Gattefossé SAS (Saint-Priest, France). The emulsifiers used were Tween®80 from Merck Schuchardt OHG (Hohenbrunn, Germany) and Pluronic®F-127 from Sigma–Aldrich Chemie Gmbh (Steinheim, Germany). The recombinant human insulin was acquired from SAFC Biosciences (Andover, UK). HPLC grade acetonitrile was from Merck (Darmstadt, Germany) and trifluoroacetic acid from Acros Organics (Morris Plains, NJ, USA) were used to perform HPLC assays. Finally, milli-Q water was produced in-house, and the rest of the reagents used were analytical grade.

3.2. Encapsulation of insulin into SLN and NLC

The lipid nanoparticles were produced using a solvent-evaporation double-emulsion modified method adapted from a previous work from our group (97). Briefly, 200 mg of lipid(s) were dissolved in 2 mL of dichloromethane, and an insulin solution of 30 mg/ml in HCl 0.1 M was poured into it. Then, the mixture was sonicated using a Bioblock Vibracell sonicator (Fisher Bioblock Scientific, Illkirch, France), during 30 seconds with 70% of amplitude. This primary emulsion was then poured into 23 ml of the surfactant solution, and sonicated again with the same conditions. The secondary emulsion was then stirred during 3 hours for organic solvent evaporation. In the experimental design, it was considered the production of SLN as controls and NLC in which a blend of solid and liquid lipids at a 70:30 ratio were dissolve in dichloromethane. In the case of SLN only the solid lipid was used. Suppocire was used as solid lipid, whereas oleic acid, capryol and miglyol were used as liquid lipids. Pluronic and Tween at concentrations of 1 and 2% (w/v) were used as surfactants. After nanoparticles production, the formulations were washed using centrifugation at 60.000 rpm during 1 hour at 4ºC and resuspended in water for further use.

3.2.1. Particle size, and zeta potential analysis

The nanoparticle samples were diluted with milli-Q water onto a suitable concentration, and the particle size, polydispersity index (PdI) and zeta potential (ZP) were evaluated using a Zetasizer Nano ZS from Malvern Panalytical Ltd (Malvern, UK). All analysis were performed in triplicate.

3.2.2. Determination of insulin association efficiency and loading capacity

The effectiveness of insulin loading into nanoparticles was evaluated by evaluating the insulin association efficiency (AE) and loading capacity (LC). They were indirectly assessed after submitting samples to centrifugation at 60,000 rpm during 1 hour at 4ºC, and the amount of insulin present in the supernatant was evaluated. Insulin was quantified by HPLC-UV using a previously reported method (98) . The analyses were performed using a XTerra®RP C18 column (Waters, Milford, MA, USA) and a LiChrospher®100 RP guard column (Merck, Darmstadt, Germany) in a Merck-Hitachi LaChrom®HPLC instrument (Merck,NJ, USA). After insulin quantification the AE and LC were determined using the following equations:

$$\mathrm{AE} = \frac{\text{Total amount of insulin } - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100 \quad (1)$$

$$\mathrm{I.C} = \frac{\text{Total amount of insulin } - \text{Free insulin supernatant}}{\text{Total dry weight of nanoaprticles}} \times 100 \quad (2)$$

4. Results and discussion

To obtain an optimal formulation of NLC to effectively load therapeutic proteins, insulin was used as model and different lipids and surfactants were used. Thus, Suppocire was used as solid lipid, and three different liquid lipids were used, namely oleic acid, capryol and miglyol. The solid and liquid lipid blend was used at a ratio of 70:30, to obtain a consistent solid matrix with the solid lipid and benefit from the advantages of the different liquid lipids. As surfactants it were used Pluronic and Tween at concentrations of 1% and 2% (w/v). The different formulations were characterized and the results are shown in Table 4.1.

The NLC optimization focused on obtaining carriers with the smallest diameter allowing to use the carriers in a wide array of delivery routes, low PdI to have homogenously dispersed carriers and negative zeta potential, characteristic of lipid nanoparticles. Overall, all formulations obtained negative zeta potential showing good colloidal stability of NLC formulations. The diameter ranged from about 130 to 500 nm with the exception of the formulation of Suppocire + Miglyol produced with Tween 2% (w/v) that obtained nanoparticles.

Table 4.1. Physicochemical characterization of unloaded NLC (n = 3, mean ± SD).

Formulations		Diameter (nm)	PdI	Zeta potential (mV)
Pluronic 1% (w/v)	Suppocire + Oleic acid	170 ± 11	0,317 ± 0,028	-23,7 ± 0,8
	Suppocire + Capryol	504 ± 20	0,508 ± 0,019	-5,9 ± 1,6
	Suppocire + Miglyol	288 ± 26	0,443 ± 0,021	-7,9 ± 1,3
Pluronic 2% (w/v)	Suppocire + Oleic acid	205 ± 25	0,273 ± 0,041	-22,8 ± 0,8
	Suppocire + Capryol	261 ± 23	0,365 ± 0,017	-4,9 ± 0,4
	Suppocire + Miglyol	305 ± 17	0,423 ± 0,021	-6,0 ± 1,6
Tween 1% (w/v)	Suppocire + Oleic acid	132 ± 5	0,402 ± 0,012	-27,2 ± 1,2
	Suppocire + Capryol	134 ± 4	0,394 ± 0,026	-25,1 ± 0,7
	Suppocire + Miglyol	499 ± 51	0,514 ± 0,046	-22,5 ± 0,5
Tween 2% (w/v)	Suppocire + Oleic acid	226 ± 35	0,577 ± 0,121	-15,1 ± 0,6
	Suppocire + Capryol	119 ± 1	0,403 ± 0,009	-18,8 ± 0,9
	Suppocire + Miglyol	1420 ± 316	0,908 ± 0,058	-21,8 ± 0,6

The formulations with the best features were then loaded with insulin and the results are shown in Table 4.2. Overall, it was shown that these formulations kept the ability to obtain the nanoparticles with good features with high drug loading. The diameter ranged from about 260 nm to 450 nm with low PdI, and the zeta potential was about -20 mV showing the good colloidal properties of the formulations. More importantly, the AE of higher than 50% which was a very good achievement regarding the hydrophilic nature of insulin loaded into the hydrophobic lipid matrices. Considering all the results, the best formulation was the NLC produced with Pluronic 1% and suppocire + oleic acid that obtained a particle size of about 275 nm, PdI of 0.300 and zeta potential of -20 mV. Regarding drug loading it was obtained an AE of 70% and LC of 2.5%. This optimization design allowed us to achieve this formulation, but the other tested formulation also obtained good results in terms of NLC features demonstrating the robustness of the production method.

Table 4.2. Physicochemical characterization of insulin-loaded NLC (n = 3, mean ± SD).

Formulations		Diameter (nm)	PdI	Zeta potential (mV)	AE (%)	LC (%)
Pluronic 1% (w/v)	Suppocire+Oleic acid	275 ± 7	0,299 ± 0,022	-20,1 ± 1,7	70,46 ± 0,27	2,47 ± 0,01
Pluronic 2% (w/v)	Suppocire+Oleic acid	262 ± 7	0,453 ± 0,019	-18,9 ± 0,8	68,98 ± 0,14	2,41 ± 0,01
Tween 1% (w/v)	Suppocire+Oleic acid	469 ± 9	0,323 ± 0,046	-22,2 ± 0,4	59,49 ± 0,16	2,08 ± 0,01
	Suppocire+Capryol	491 ± 14	0,261 ± 0,021	-22,1 ± 0,4	71,01 ± 1,98	2,49 ± 0,07
Tween 2% (w/v)	Suppocire+Oleic acid	391 ± 9	0,301 ± 0,013	-18,8 ± 0,1	53,63 ± 0,03	1,88 ± 0,01
	Suppocire+Capryol	446 ± 14	0,265 ± 0,011	-20,1 ± 0,5	64,71 ± 0,89	2,26 ± 0,03

The optimized formulation was further produced with a higher amount of insulin placing 30 mg inside the 200 mg of lipid matrices, and the formulation was characterized (Table 2.3). Formulations of SLN using the same production protocol but without placing the liquid lipid, and unloaded carriers were used as controls. It was observed that all formulations obtained a particle size of 200 nm and good PdI demonstrating again the robustness of the production method. Also, it was observed an increase in zeta potential when insulin was encapsulated in comparison to unloaded carriers. This is mainly due to the presence of insulin adsorbed on the carriers surface. More importantly, it was observed that the NLC had the higher AE compared with the SLN, obtaining an AE of about 85% and LC of 11%, which is a remarkable achievement.

It is foreseen the use of this optimized formulation in different studies, especially on evaluating the structure of bioactivity of therapeutic proteins loaded into NLC.

Table 4.3. Physicochemical characterization of the optimized formulation of insulin-loaded NLC. The nanocarriers were produced with suppocire and oleic acid as lipids, and pluronic 1% (w/v) as surfactant (n = 3, mean ± SD).

Formulations		Diameter (nm)	PdI	Zeta Potential (mV)	AE (%)	LC (%)
SLN	Unloaded	201 ± 20	0,367 ± 0,055	-17,1 ± 2,3	-	-
	Loaded	201 ± 8	0,196 ± 0,017	-8,3 ± 0,7	70.26 ± 2,27	6.22 ± 0.57
NLC	Unloaded	194 ± 25	0,329 ± 0,023	-28,1 ± 1,4	-	-
	Loaded	203 ± 5	0,217 ± 0,015	-18,4 ± 0,7	85.33 ± 3.25	10.57 ± 4.44

5. Conclusions

The use of proteins as therapeutics has significantly improved the treatment of several diseases, redefining the shape of several medical fields. Therapeutic proteins are extremely valuable as therapeutics and present a wide range of advantages. Mitigating the major challenges of the administration of therapeutic proteins will allow a new range of opportunities in the medical field. The use of lipid nanoparticles has the potential to overcome the delivery challenges of therapeutic proteins, ultimately allowing their administration throw different administrations routes. Therefore, the objective of this work was first to give an overview of the state of the art of the encapsulation of therapeutic proteins into SLN and NLC. Then, bearing in mind the advantages of NLC over the SLN, an optimized administration method for the encapsulation of therapeutic proteins, avoiding stress conditions that could affect the protein structure, was developed.

Therefore, using insulin as a model protein, the lipid nanoparticles were produced using a solvent-evaporation double emulsion modified method. The results of unloaded nanoparticles diameter ranged from 260 nm to 450 nm, with low PdI and zeta potential above -20 mV. The insulin loaded formulations had a mean diameter of 200 nm and good PdI. These results demonstrate the robustness of the production method. Finally, comparing the insulin-loaded NLC with the insulin-loaded SLN, the first ones had the higher AE of 85% and higher LC of 11%. Therefore, these are very promising results indicating that the optimized formulation can be used in future *in vivo* studies to evaluate the structure and bioactivity of therapeutic proteins encapsulated into NLC.

6. References:

1. Martins S, Sarmento B, Ferreira DC, Souto EB. Lipid-based colloidal carriers for peptide and protein delivery--liposomes versus lipid nanoparticles. International journal of nanomedicine. 2007;2(4):595–607.
2. Desai N. Challenges in development of nanoparticle-based therapeutics. The AAPS journal. 2012;14(2):282–95.
3. Navalakhe RM, Nandedkar TD. Application of nanotechnology in biomedicine. Indian journal of experimental biology. 2007;45(2):160–5.
4. Suri SS, Fenniri H, Singh B. Nanotechnology-based drug delivery systems. Journal of Occupational Medicine and Toxicology. 2007;2(1):16.
5. Has C, Sunthar P. A comprehensive review on recent preparation techniques of liposomes. Journal of Liposome Research. 2020 Oct 1;30(4):336–65.
6. Shah S, Dhawan V, Holm R, Nagarsenker MS, Perrie Y. Liposomes: Advancements and innovation in the manufacturing process. Advanced Drug Delivery Reviews. 2020 Jan 1;154–155:102–22.
7. MÃ¼ller R. Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art. European Journal of Pharmaceutics and Biopharmaceutics. 2000 Jul 3;50(1):161–77.
8. Almeida AJ, Souto E. Solid lipid nanoparticles as a drug delivery system for peptides and proteins. Advanced Drug Delivery Reviews. 2007 Jul 10;59(6):478–90.
9. HARTLEY H. Origin of the Word ‘Protein.’ Nature. 1951 Aug;168(4267):244–244.
10. Dimitrov DS. Therapeutic Proteins. In 2012. p. 1–26.
11. Dingman R, Balu-Iyer S v. Immunogenicity of Protein Pharmaceuticals. Journal of Pharmaceutical Sciences. 2019 May 1;108(5):1637–54.
12. Fonte P, Sousa F, Sarmento B. Polyester-Based Nanoparticles for Delivery of Therapeutic Proteins. In 2018. p. 255–74.
13. Weiss M, Steiner DF, Philipson LH. Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships. Endotext. 2000.
14. Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. Nature Reviews Drug Discovery. 2008 Jan;7(1):21–39.
15. Ozer S, Kerimoglu O, Ugurlu T. Nanocarriers: Novel Approaches to Oral Delivery of Insulin. Clinical and Experimental Health Sciences. 2017 Sep 19;7(3):115–22.
16. Patel S, Dutta S. Insulin. RCSB Protein Data Bank. 2018 Aug 1.
17. Craik DJ, Fairlie DP, Liras S, Price D. The Future of Peptide-based Drugs. Chemical Biology & Drug Design. 2013 Jan;81(1):136–47.
18. BCC Research. Global Markets for Bioengineered Protein Drugs. 2016.
19. Zaman R, Islam RA, Ibnat N, Othman I, Zaini A, Lee CY, et al. Current strategies in extending half-lives of therapeutic proteins. Journal of Controlled Release. 2017 May;301:477–94.
20. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nature Reviews Drug Discovery. 2005 Apr;4(4):298–306.
21. Geraldes DC, Beraldo-de-Araújo VL, Pardo BOP, Pessoa Junior A, Stephano MA, de Oliveira-Nascimento L. Protein drug delivery: current dosage form profile and formulation strategies. Journal of Drug Targeting. 2020 Apr 20;28(4):339–55.

22. Carter PJ. Introduction to current and future protein therapeutics: A protein engineering perspective. Experimental Cell Research. 2011 May 15;317(9):1261–9.
23. Goldman B, DeFrancesco L. The cancer vaccine roller coaster. Nature Biotechnology. 2009 Feb;27(2):129–39.
24. Reichert JM. Trends in development and approval times for new therapeutics in the United States. Nature Reviews Drug Discovery. 2003 Sep;2(9):695–702.
25. Biotechnology Innovation Organization, Pharma Intelligence Informa, Quantitative Life Sciences. Clinical Development Success Rates and Contributing Factors 2011–2020. 2021.
26. Matthews H, Hanison J, Nirmalan N. "Omics"-Informed Drug and Biomarker Discovery: Opportunities, Challenges and Future Perspectives. Proteomes. 2016 Sep 12;4(3):28.
27. Werle M, Bernkop-Schnürch A. Strategies to improve plasma half life time of peptide and protein drugs. Amino Acids. 2006 Jun 20;30(4):351–67.
28. Murray JE, Laurieri N, Delgoda R. Proteins. Pharmacognosy: Fundamentals, Applications and Strategy. 2017 Jan 1;477–94.
29. Baker MP, Reynolds HM, Lumicisi B, Bryson CJ. Immunogenicity of protein therapeutics: The key causes, consequences and challenges. Self/nonself. 2010 Oct;1(4):314–22.
30. Braun A, Kwee L, Labow MA, Alsenz J. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. Pharmaceutical research. 1997 Oct;14(10):1472–8.
31. Moussa EM, Panchal JP, Moorthy BS, Blum JS, Joubert MK, Narhi LO, et al. Immunogenicity of Therapeutic Protein Aggregates. Journal of pharmaceutical sciences. 2016 Feb;105(2):417–30.
32. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharmaceutical research. 2003 Sep;20(9):1325–36.
33. Roberts CJ. Non-native protein aggregation kinetics. Biotechnology and Bioengineering. 2007 Dec 1;98(5):927–38.
34. Mahler H-C, Friess W, Grauschopf U, Kiese S. Protein aggregation: Pathways, induction factors and analysis. Journal of Pharmaceutical Sciences. 2009 Sep;98(9):2909–34.
35. van Beers MMC, Sauerborn M, Gilli F, Brinks V, Schellekens H, Jiskoot W. Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. Pharmaceutical research. 2011 Oct;28(10):2393–402.
36. Fan J, de Lannoy IAM. Pharmacokinetics. Biochemical Pharmacology. 2014 Jan 1;87(1):93–120.
37. Kontermann RE. Half-Life Modulating Strategies-An Introduction. In: Therapeutic Proteins. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2012. p. 1–21.
38. Leeson P. Chemical beauty contest. Nature. 2012 Jan 25;481(7382):455–6.
39. Fan J, de Lannoy IAM. Pharmacokinetics. Biochemical Pharmacology. 2014 Jan 1;87(1):93–120.
40. Conner KP, Devanaboyina SC, Thomas VA, Rock DA. The biodistribution of therapeutic proteins: Mechanism, implications for pharmacokinetics, and methods of evaluation. Pharmacology & therapeutics. 2020;212:107574.

41. Yan Q, Huang M, Lewis MJ, Hu P. Structure Based Prediction of Asparagine Deamidation Propensity in Monoclonal Antibodies. mAbs. 2018 Aug 18;10(6):901–12.
42. Butreddy A, Janga KY, Ajjarapu S, Sarabu S, Dudhipala N. Instability of therapeutic proteins — An overview of stresses, stabilization mechanisms and analytical techniques involved in lyophilized proteins. International Journal of Biological Macromolecules. 2021 Jan 15;167:309–25.
43. Meissner J, Prause A, Bharti B, Findenegg GH. Characterization of protein adsorption onto silica nanoparticles: influence of pH and ionic strength. Colloid and Polymer Science. 2015 Nov 11;293(11):3381–91.
44. Shirwaikar A, Srinivasan K, Alex J, Prabu S, Mahalaxmi R, Kumar R, et al. Stability of proteins in aqueous solution and solid state. Indian Journal of Pharmaceutical Sciences. 2006;68(2):154.
45. Butreddy A, Janga KY, Ajjarapu S, Sarabu S, Dudhipala N. Instability of therapeutic proteins — An overview of stresses, stabilization mechanisms and analytical techniques involved in lyophilized proteins. International Journal of Biological Macromolecules. 2021 Jan 15;167:309–25.
46. Jo M, Ko S, Hwang B, Min S, Ha JY, Lee JC, et al. Engineered human FcγRIIa fusion: A novel strategy to extend serum half-life of therapeutic proteins. Biotechnology and Bioengineering. 2020 Aug 16;117(8):2351–61.
47. JevsÌŒevar S, Kunstelj M, Porekar VG. PEGylation of therapeutic proteins. Biotechnology Journal. 2010 Jan;5(1):113–28.
48. Veronese FM, Mero A. The Impact of PEGylation on Biological Therapies. BioDrugs. 2008;22(5):315–29.
49. Bayda S, Adeel M, Tuccinardi T, Cordani M, Rizzolio F. The History of Nanoscience and Nanotechnology: From Chemical-Physical Applications to Nanomedicine. Molecules (Basel, Switzerland). 2019 Dec 27;25(1).
50. National Nanotechnology Initiative. What Is Nanotechnology?. 2021 [cited 2021 Sep 4]. Available from: https://www.nano.gov/nanotech-101/what/definition
51. Jeevanandam J, Barhoum A, Chan YS, Dufresne A, Danquah MK. Review on nanoparticles and nanostructured materials: history, sources, toxicity and regulations. Beilstein journal of nanotechnology. 2018;9:1050–74.
52. Dean S, Mansoori G, Fauzi Soelaiman T. Nanotechnology — An Introduction for the Standards Community. Journal of ASTM International. 2005;2(6).
53. NanoBiologics Research Corporation. Nanologics_Nanoscale. 2017.
54. Feynman R. There’s Plenty of Room at the Bottom. In 1960. p. 22–36.
55. Bayda S, Adeel M, Tuccinardi T, Cordani M, Rizzolio F. The History of Nanoscience and Nanotechnology: From Chemical–Physical Applications to Nanomedicine. Molecules. 2019 Dec 27;25(1).
56. Xue H, Li J, Xie H, Wang Y. Review of Drug Repositioning Approaches and Resources. Int J Biol Sci. 2018;14(10):1232–44.
57. Mehnert W. Solid lipid nanoparticles Production, characterization and applications. Advanced Drug Delivery Reviews. 2001 Apr 25;47(2–3):165–96.
58. de Jong. Drug delivery and nanoparticles: Applications and hazards. International Journal of Nanomedicine. 2008 Jun;133.
59. Saallah S, Lenggoro IW. Nanoparticles Carrying Biological Molecules: Recent Advances and Applications. KONA Powder and Particle Journal. 2018;35:89–111.
60. DeFrates K, Markiewicz T, Gallo P, Rack A, Weyhmiller A, Jarmusik B, et al. Protein Polymer-Based Nanoparticles: Fabrication and Medical Applications. International Journal of Molecular Sciences. 2018 Jun 9;19(6):1717.

61. Jeoghoon S, Michaels M. Challenges in Protein Product Development. Warne NW, Mahler H-C, editors. Cham: Springer International Publishing; 2018. 536–542. (AAPS Advances in the Pharmaceutical Sciences Series; vol. 38).
62. Astefanei A, Núñez O, Galceran MT. Characterisation and determination of fullerenes: A critical review. Analytica Chimica Acta. 2015 Jul 2;882:1–21.
63. Khan I, Saeed K, Khan I. Nanoparticles: Properties, applications and toxicities. Arabian Journal of Chemistry. 2019 Nov 1;12(7):908–31.
64. Ngoy JM, Wagner N, Riboldi L, Bolland O. A CO2 Capture Technology Using Multi-walled Carbon Nanotubes with Polyaspartamide Surfactant. Energy Procedia. 2014 Jan 1;63:2230–48.
65. Abd Ellah NH, Abouelmagd SA. Surface functionalization of polymeric nanoparticles for tumor drug delivery: approaches and challenges. Expert Opinion on Drug Delivery. 2017 Feb 1;14(2):201–14.
66. Mansha M, Khan I, Ullah N, Qurashi A. Synthesis, characterization and visible-light-driven photoelectrochemical hydrogen evolution reaction of carbazole-containing conjugated polymers. International Journal of Hydrogen Energy. 2017 Apr 20;42(16):10952–61.
67. Müller RH, Radtke M, Wissing SA. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Advanced Drug Delivery Reviews. 2002 Nov;54:S131–55.
68. Brahmankar D, Jaiswal SB. Biopharmaceutics and pharmacokinetics : a treatise. 1st editio. Dehli : Vallabh Prakashan, 2005 ©1995, editor. Delhi: Vallabh Prakashan Publishers; 1995. 296–297.
69. Gunasekaran T, Haile T, Nigusse T, Dhanaraju MD. Nanotechnology: an effective tool for enhancing bioavailability and bioactivity of phytomedicine. Asian Pacific journal of tropical biomedicine. 2014 May;4(Suppl 1):S1-7.
70. Ganesan P, Narayanasamy D. Lipid nanoparticles: Different preparation techniques, characterization, hurdles, and strategies for the production of solid lipid nanoparticles and nanostructured lipid carriers for oral drug delivery. Sustainable Chemistry and Pharmacy. 2017 Dec;6:37–56.
71. Patel D, Kesharwani R, Gupta S. Development & Screenning Approach For Lipid Nanoparticle : A Review. International Journal of Innovations in Pharmaceutical Sciences. 2013;2:27–32.
72. Souto EB, Baldim I, Oliveira WP, Rao R, Yadav N, Gama FM, et al. SLN and NLC for topical, dermal, and transdermal drug delivery. Expert Opinion on Drug Delivery. 2020 Mar 3;17(3):357–77.
73. Schwarz C, Mehnert W, Lucks JS, Müller RH. Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterization and sterilization. Journal of Controlled Release. 1994 Apr 1;30(1):83–96.
74. Morel S, Terreno E, Ugazio E, Aime S, Gasco MR. NMR relaxometric investigations of solid lipid nanoparticles (SLN) containing gadolinium(III) complexes. European Journal of Pharmaceutics and Biopharmaceutics. 1998 Mar 1;45(2):157–63.
75. Joshi MD, Müller RH. Lipid nanoparticles for parenteral delivery of actives. European Journal of Pharmaceutics and Biopharmaceutics. 2009 Feb;71(2):161–72.
76. Borges A, de Freitas V, Mateus N, Fernandes I, Oliveira J. Solid Lipid Nanoparticles as Carriers of Natural Phenolic Compounds. Antioxidants. 2020 Oct 15;9(10):998.

77. Muchow M, Maincent P, Müller RH. Lipid Nanoparticles with a Solid Matrix (SLN®, NLC®, LDC®) for Oral Drug Delivery. Drug Development and Industrial Pharmacy. 2008 Jan 20;34(12):1394–405.
78. Radomska-Soukharev A, Müller RH. Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading capacity and sustained release potential. Journal of Controlled Release. 1997 Oct 13;48(2–3):223–36.
79. Schubert MA, Müller-Goymann CC. Characterisation of surface-modified solid lipid nanoparticles (SLN): Influence of lecithin and nonionic emulsifier. European Journal of Pharmaceutics and Biopharmaceutics. 2005 Sep 1;61(1–2):77–86.
80. Almeida AJ, Runge S, Müller RH. Peptide-loaded solid lipid nanoparticles (SLN): Influence of production parameters. International Journal of Pharmaceutics. 1997 Apr 28;149(2):255–65.
81. Aryani NLD, Siswandono, Soeratri W, Rahmasari FP, Sari DRK. Experimental Development and Molecular Docking: Nanostructured Lipid Carriers (NLCs) of Coenzyme Q10 Using Stearic Acid and Different Liquid Lipids as Lipid Matrix. International Journal of Applied Pharmaceutics. 2021 Jan 7;108-16.
82. Joshi MD, Prabhu RH, Patravale VB. Fabrication of Nanostructured Lipid Carriers (NLC)-Based Gels from Microemulsion Template for Delivery Through Skin. In 2019. p. 279–92.
83. Shidhaye S, Vaidya R, Sutar S, Patwardhan A, Kadam V. Solid Lipid Nanoparticles and Nanostructured Lipid Carriers – Innovative Generations of Solid Lipid Carriers. Current Drug Delivery [Internet]. 2008 Oct 1;5(4):324–31.
84. Sandhya M, Ramasamy D, Sudhakar K, Kadirgama K, Harun WSW. Ultrasonication an intensifying tool for preparation of stable nanofluids and study the time influence on distinct properties of graphene nanofluids – A systematic overview. Ultrasonics Sonochemistry. 2021 May 1;73:105479.
85. Siddiqui A, Alayoubi A, El-Malah Y, Nazzal S. Modeling the effect of sonication parameters on size and dispersion temperature of solid lipid nanoparticles (SLNs) by response surface methodology (RSM). Pharmaceutical Development and Technology. 2014 May 16;19(3):342–6.
86. Yang R, Gao R, Li F, He H, Tang X. The influence of lipid characteristics on the formation, in vitro release, and in vivo absorption of protein-loaded SLN prepared by the double emulsion process. Drug Development and Industrial Pharmacy. 2011 Feb 25;37(2):139–48.
87. Yang R, Gao R-C, Cai C-F, Xu H, Li F, He H-B, et al. Preparation of Gel-Core-Solid Lipid Nanoparticle: A Novel Way to Improve the Encapsulation of Protein and Peptide. Chemical and Pharmaceutical Bulletin. 2010;58(9):1195–202.
88. Gasco MR. Solid lipid microspheres having a narrow size distribution and method for producing them. 91113152.2, 1993. p. 7.
89. Morel S, Ugazio E, Cavalli R, Gasco MR. Thymopentin in solid lipid nanoparticles. International Journal of Pharmaceutics. 1996 Apr 30;132(1–2):259–61.
90. Morel S, Rosa Gasco M, Cavalli R. Incorporation in lipospheres of [d-Trp-6]LHRH. International Journal of Pharmaceutics. 1994 May 2;105(2):R1–3.
91. Almeida AJ, Souto E. Solid lipid nanoparticles as a drug delivery system for peptides and proteins. Advanced Drug Delivery Reviews. 2007 Jul 10;59(6):478–90.
92. Sjöström B, Bergenståhl B. Preparation of submicron drug particles in lecithin-stabilized o/w emulsions I. Model studies of the precipitation of cholesteryl acetate. International Journal of Pharmaceutics. 1992 Dec 8;88(1–3):53–62.

93. Quintanar-Guerrero D, Tamayo-Esquivel D, Ganem-Quintanar A, Allémann E, Doelker E. Adaptation and optimization of the emulsification-diffusion technique to prepare lipidic nanospheres. European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical Sciences
94. He H, Wang P, Cai C, Yang R, Tang X. VB12-coated Gel-Core-SLN containing insulin: Another way to improve oral absorption. International Journal of Pharmaceutics. 2015 Sep 30;493(1–2):451–9.
95. Schubert MA, Müller-Goymann CC. Solvent injection as a new approach for manufacturing lipid nanoparticles – evaluation of the method and process parameters. European Journal of Pharmaceutics and Biopharmaceutics. 2003 Jan 1;55(1):125–31.
96. Schubert M. Solvent injection as a new approach for manufacturing lipid nanoparticles – evaluation of the method and process parameters. European Journal of Pharmaceutics and Biopharmaceutics. 2003 Jan;55(1):125–31.
97. Fonte P, Araújo F, Seabra V, Reis S, van de Weert M, Sarmento B. Co-encapsulation of lyoprotectants improves the stability of protein-loaded PLGA nanoparticles upon lyophilization. International Journal of Pharmaceutics. 2015 Dec;496(2):850–62.
98. Sarmento B, Ribeiro A, Veiga F, Ferreira D. Development and validation of a rapid reversed-phase HPLC method for the determination of insulin from nanoparticulate systems. Biomedical Chromatography. 2006 Sep;20(9):898–903.