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Review article

An insight on lipid nanoparticles for therapeutic proteins delivery

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ABSTRACT

Therapeutic proteins are well-tolerated bioactive compounds used in different therapies, due to its high specificity and biopotency. Nevertheless, they may also present some physicochemical instability, leading to loss of bioactivity hampering treatments. This can be avoided by its loading into lipid nanoparticles, which are biocompatible and biodegradable carriers. The use of lipids nanoparticles to deliver therapeutic proteins overcomes different challenges, allowing its administration by all delivery routes. Thus, therapeutic proteins may be loaded into liposomes, the first developed lipid-based nanocarriers composed of phospholipid bilayers, solid lipid nanoparticles composed of a solid lipid matrix, or nanostructured lipid carriers made of a blend of liquid and solid lipid as matrix. The latter are currently marking the trend in lipid nanocarriers due to its high loading capacity, good stability upon storage and better sustained release pattern. Production methods must focus both on attaining the desired nanocarrier features, and maintenance of therapeutic proteins structure and bioactivity. This review aims to make an insight overview on the application of lipid nanoparticles to deliver therapeutic proteins, showing its potential in different therapies. A special focus is given to the production techniques to obtain therapeutic proteins-loaded lipid nanoparticles.

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 [1]. 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.

Nanotechnology allows the development 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 them 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 delivery of therapeutic proteins, nanoparticles allow overcoming its delivery challenges [3,4].

In the last years, lipid nanoparticles have been studied as drug delivery systems, as an attempt to overcome the problems and improve the characteristics of therapeutic proteins, protecting it from degradation *in vivo*, allowing a controlled release, modifying biodistribution, and enhancing targeted delivery, solubility, and bioavailability. The use of lipids in the development of delivery systems started with phospholipid vesicles named as "liposomes" in 1965 by Prof. A.D. Bangham. Liposomes are spherical vesicular systems, composed of one or multiple phospholipid bilayers entrapping an aqueous phase, firstly introduced in the cosmetic market in 1986, and after in pharmaceutical products at the

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List of abbreviations and acronyms

ADME	Absorption, distribution, metabolism, and excretion
Ala	Alanine
BSA	Bovine serum albumin
CNT	Carbon nanotubes
DNA	Deoxyribonucleic acid
EE	Encapsulation efficiency
GCSLN	Gel core solid lipid nanoparticles
GRAS	Generally recognized as safe
HLB	Hydrophilic-lipophilic balance
HPH	Hot pressure homogenization
LC	Loading capacity
MLV	Multilamellar vesicle
NLC	Nanostructured lipid carriers
OLV	Oligolamellar vesicle
PdI	Polydispersity index
PEG	Polyethylene glycol
pI	Isoelectric point
SLN	Solid lipid nanoparticles
The	Threonine
ULV	Unilamellar vesicle
Val	Valine
VB12	Vitamin B12

end of the 1980s [5–7]. A few years later, it was developed a new generation of lipid nanoparticles, the solid lipid nanoparticles (SLN), and even later the nanostructured lipid carrier (NLC) made. The SLN are composed of a solid lipid matrix of biodegradable and biocompatible lipid or blend of lipids. The NLC 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 undesired expulsion of the encapsulated drug during storage. By presenting a matrix composed of two different lipids, the NLC matrix presents imperfections that increase their loading capacity, better accommodating the encapsulated drug, avoiding drug expulsion during storage, and allowing better sustained released properties compared to SLN [5–7]. Both SLN and NLC use similar 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 structure of the protein and compromise its bioactivity [1,8].

Overall, this review aims to perform an overview on the application of lipid nanoparticles to deliver therapeutic proteins in different therapies. A special insight is given in the production methods to obtain the different therapeutic protein-loaded lipid nanoparticles.

2. Therapeutic proteins

Jöns Jakob Berzelius first used the term "protein" in 1838. These molecules were identified in the 18th century as having specific biological properties, namely the ability to coagulate when treated with heat or acid [9]. Currently, there are over 250 proteins used clinically for different purposes from prophylaxis 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 disulphide bonds and an additional intrachain disulphide bond present in the A-chain, as shown in Fig. 1 [13]. In 1922 insulin was first purified from bovine and porcine pancreas, and used for treatment of diabetic patients, emerging as a life-saving treatment [14]. Nonetheless, with the widespread use of this protein, some problems became known: the limited availability of animal pancreas for purification of insulin which would not be sufficient for the daily treatments of patients, the cost associated with the process, and the immunological reactions developed by some patients. To solve such 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 enormous quantities of this bacteria, the large-scale production of human insulin was accomplished and, in 1982, recombinant insulin was

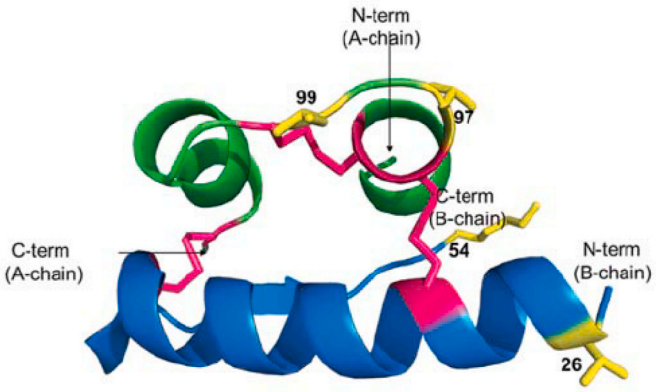

Fig. 1. Tridimensional structure of human insulin. A-chain, in green, is covalently connected via disulphide bonds, in pink, to B-Chain, in blue. *Reprinted with permission from* Ref. [15]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

approved by the Food and Drug Administration (FDA), representing one of the biggest achievements of modern healthcare science [14].

Recombinant DNA technology established on an industrial scale has dramatically escalated the number of biotechnology drugs approved and under investigation. According to 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 have reached $228.4 billion by 2021. In terms of the annual growth rate, in 2016 was $39.8 billion and it is expected to have reached $40.2 billion in 2021, which is about 10% of the ethical pharmaceutical market [16,17]. 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 [12,14,18–21].

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 extremely 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 particularly 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

Table 1
Classification of therapeutic proteins by pharmacologic activity. *Adapted from* Refs. [14,22].

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

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 [23]. 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 [24]. In Fig. 2 it is represented the duration of the phases of development for therapeutic proteins by disease, and in Fig. 3 for synthetic drugs. Moreover, due to their singularity in terms of form and function, companies can obtain far-reaching patent protection [14].

Nonetheless, as shown in Table 2, despite all the advantages, the administration of protein drugs still represents a challenge, due to their immunogenicity problems, poor bioavailability due to their physico-chemical instability and consequent fast degradation in serum, production challenges and difficulty to permeate membranes as the gastrointestinal epithelium. Since therapeutic proteins suffer rapid degradation in serum and fast elimination, they are usually administered 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 leads to side effects [18,26]. To diminish those side effects and address their narrow therapeutic ranges, several approaches have been developed and evaluated to extend the therapeutical proteins half-life in circulation. By extending the proteins half-life, both problems mentioned would be addressed, maintaining the therapeutical concentrations with lower doses [18,26,27].

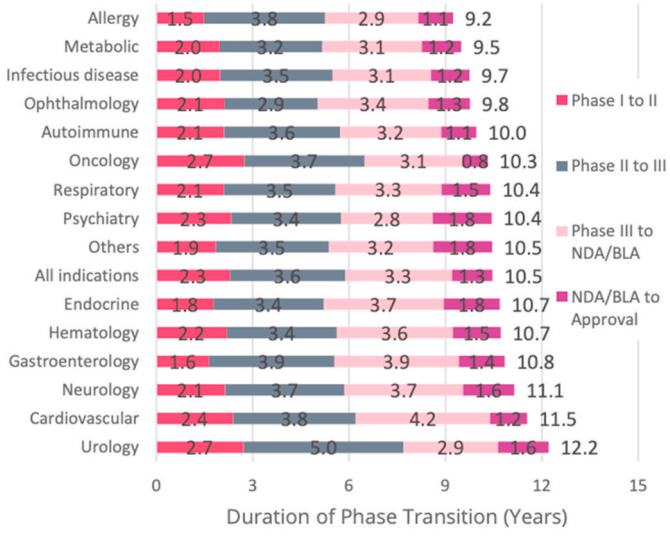

Fig. 2. Timeline for product development of therapeutic proteins by disease. These results are based on 6151 successful phase transitions in the 2011–2020 period. *Reprinted from* Ref. [24].

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 its administration problems are crucial to increase the number of formulations reaching the pharmaceutical market [11,18].

3. Delivery challenges of therapeutic proteins

As previously mentioned, therapeutic proteins have delivery challenges that compromise their therapeutic effect and limits delivery 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.

3.1. Immunogenicity

The development of therapeutic proteins was followed by the expectation that the same as the "self" derived proteins, they would avoid immunogenicity. 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 effect on the protein, reducing the protein half-life or triggering 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 proteins [28].

There is also a relationship between aggregated proteins and enhanced immunogenicity, with studies showing this correlation in a variety of models [28]. 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 [29–33]. According to a study developed by Braun et al. (1997), 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 [34].

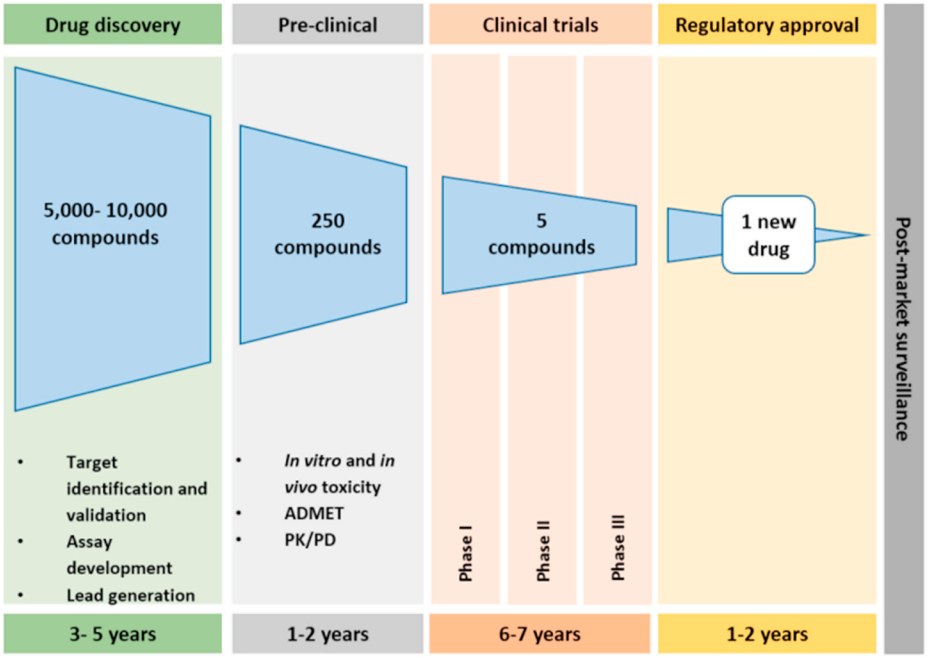

Fig. 3. Product development timeline for synthetic drugs. It takes on average approximately 15 years for a synthetic drug to reach the market. *Reprinted from* Ref. [25].

Table 2
Advantages and disadvantages of therapeutic proteins. *Adapted from* Refs. [14,16].

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

3.2. Short half-life

Pharmacokinetics is, by definition, the study of the movement of xenobiotics (drugs/compounds/chemical entities) within the body after administration, being affected by four distinct, yet interrelated processes: absorption, distribution, metabolism, and excretion (ADME) [35]. The efficacy of therapeutical proteins is significantly affected by their pharmacokinetic properties as their plasma half-life [36].

Since most of the activity of the endogenous protein resembles hormones activity, they frequently present fast serum elimination, which is desirable from the hormonal regulation point of view. Nonetheless, therapeutic proteins are 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 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 [35,37,38]. 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 hydrolysed 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. (2018) 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 [39]. The results showed that the difference in asparagine deamidation rate could be due to structure conformation, structure flexibility, and solvent accessibility [40].

3.3. Isoelectric point and 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 [38,40].

A study on the characterization of protein adsorption onto nanoparticles, highlighted the impact of isoelectric interactions on globular proteins Lyz and β-Lg onto negatively charged 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 molecules. For pH values closer to pI the protein-protein interactions were less relevant which extended the adsorption significantly above one monolayer [41].

3.4. 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 disulphide exchange. Fig. 4 represents the different physical and chemical sources of protein instability [40,42].

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 administered 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 specific physicochemical proprieties as lipophilicity, molecular weight, size, and surface charge that influence the pharmacokinetics and biodistribution of the protein.

There are several approaches to overcome the therapeutic protein delivery challenges, which 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 protein chain, which has been proven to reduce immunogenicity and proteolytic cleavage *in vivo*. Considering that the immunoglobulin G has a long-circulating serum half-life (~3 weeks) through pH-dependent FcRn binding-mediated recycling, a study was performed 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 suggests that this strategy has the potential to prolong the half-life of therapeutic proteins [43]. 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 [44]. 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 (Fig. 5A). PEG main advantages are its solubility in both aqueous and organic solvents, presenting great flexibility, high hydration that consequently increases its hydrodynamic volume, and a range of molecular weight species allowing tuneable 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 may increase solubility, become resistant to antibodies, proteolytic enzymes, and cells, and, due to their increased size, are more slowly filtered by the kidneys [45].

The main foundation of bioconjugation approaches is that during hepatic metabolism, proteins are taken by hepatocytes, 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

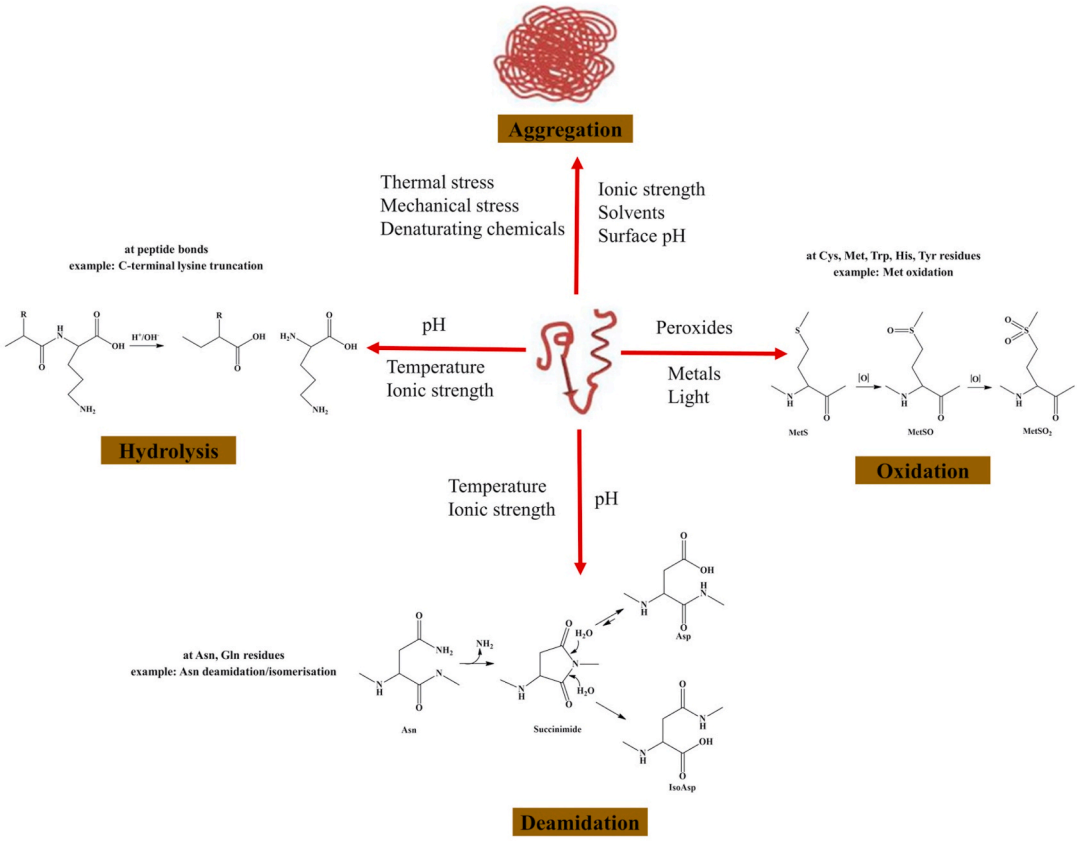

Fig. 4. Physical and chemical instability sources of therapeutic proteins. *Reprinted with permission from* Ref. [40].

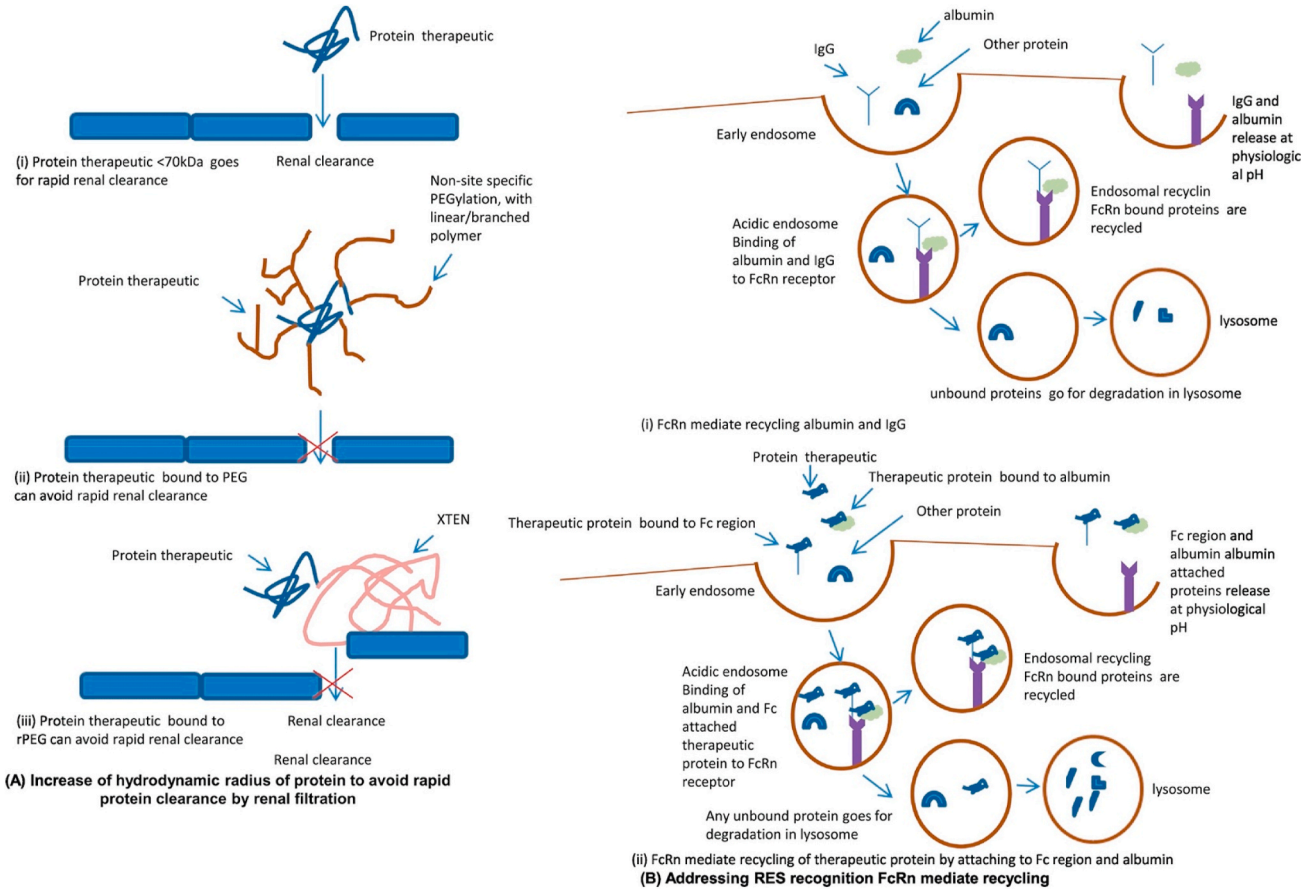

Fig. 5. Protein half-life extension by avoidance of rapid renal clearance by pegylation (A) and receptor-mediated recycling (B). *Reprinted with permission from* Ref. [18].

therapeutic proteins (Fig. 5 B) [44].

Carriers are used to protect the protein allowing its targeted and controlled delivery [44]. The use of nanocarriers to deliver therapeutic proteins is deepened in the following section.

4. 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 [46,47]. 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 [48]. The prefix "nano" derives from the Greek word that means "dwarf" or reduction in size, corresponding to a one thousand million 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 [49].

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 vol 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 [50]. 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 enormous 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 [46].

Drug discovery is a time-consuming, arduous, expensive, and high-risk process, with a significantly low success rate and several challenges to overcome. Furthermore, in the last decades, it became evident that drug development alone is not enough 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 pharmacodynamics 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 [51,52].

Drug delivery systems are used to enable controllable drug release and improve both its safety and efficacy. 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 nanotechnologies include more specific drug targeting and delivery, reducing toxicity while maintaining therapeutic effects, enhancing safety and biocompatibility, and accelerating the new medicines development process. Even though drug delivery systems do not modify the pharmacokinetic or pharmacodynamics 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 [18,53].

Nanoparticles are attractive as drug delivery systems due to their

unique characteristics as the surface to mass ratio is 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 and the enhanced solubility and diffusivity have been proven to increase the blood circulation half-life [45]. As mentioned, the size of the nanoparticles ranges from 1 to 1000 nm, but for nanomedicine purposes, sizes smaller than 200 nm are preferable due to the ability to traverse micro-capillaries. Still, particle sizes above 100 nm may be required for loading enough drugs [53,54]. 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 [18,55].

There are several classifications of nanoparticles according to their morphology, size, and chemical properties (Fig. 6). Considering the composition materials of the nanoparticles they can be divided into 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 like a rolling graphite sheet. These are frequently used for commercial applications fillers and efficient gas adsorbents for environmental purposes [56–58].
(2) Metallic nanoparticles – Made by metals precursors with unique optoelectrical properties which make them valuable for applications in research areas [57].
(3) Polymeric nanoparticles – Usually organic-based nanoparticles, in their majority with nanosphere or nanocapsules shape with a wide range of applications [59,60].
(4) Lipid-based nanoparticles – Made of lipids both in a solid or liquid state. It is fully addressed in the next section.

The latter are the focus of this review and are fully disclosed in the next section.

5. Lipid nanoparticles for therapeutic proteins delivery

The use of lipid nanoparticles as drug carriers have been studied for years. The first emulsion introduced as carrier systems, in the fifties, was only intended to reduce the drug side effects. Although accomplishing the intended goal, they did not have the expected success, which can be explained by the physical instability caused by the incorporated drug and the low solubility of the used lipids. Later, in 1965, liposomes were developed by Bangham and introduced as drug delivery systems in 1986 by Dior® in the cosmetic market. Few years later, at the end of the eighties, liposomes started being used in the 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 excessive cost of pharmaceutical liposomes [62].

Other lipid based systems for encapsulation of therapeutic drugs have been extensively used specifically for topical drug administration once their lipid bilayers mimic the human cell membrane, to the delivery of cosmetics like vegetable oils and therapeutic factors that promote wound healing [63–65]. They are used also for oral delivery to encapsulate unstable compounds like antimicrobials, antioxidants, flavors, and bioactive elements to preserve their functionality [64,66]. Finally, the advantage of ocular therapy by topical administration is not

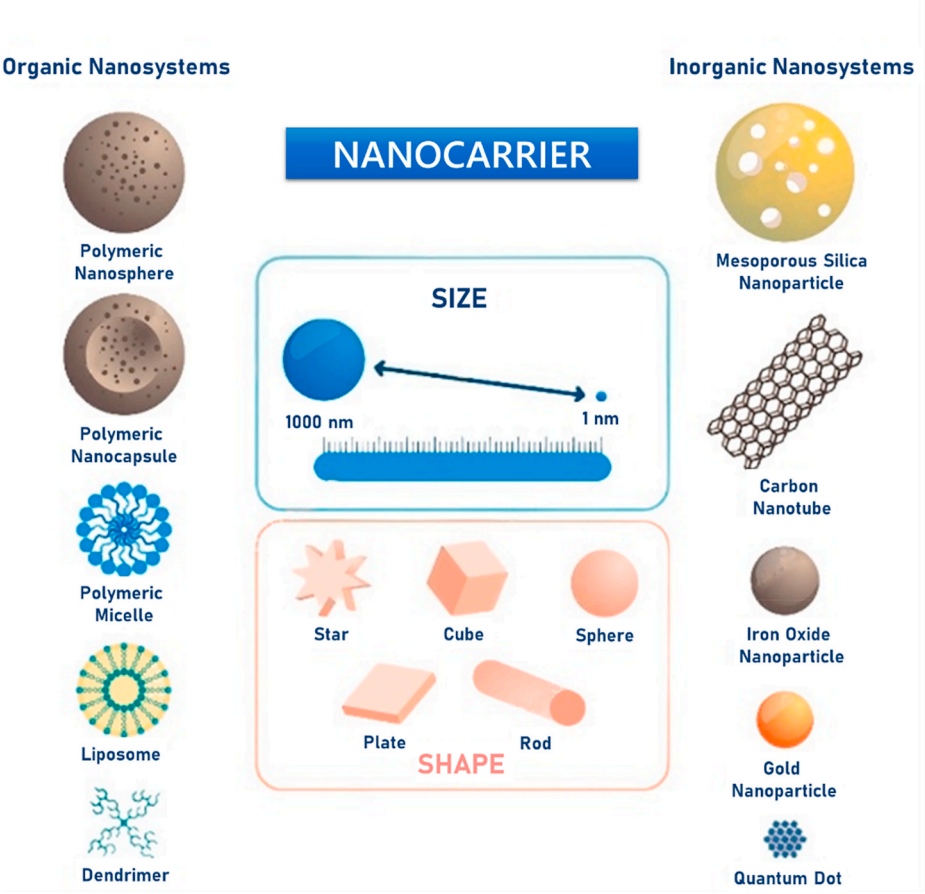

Fig. 6. Types, size range and shapes of nanocarriers. *Reprinted from* Ref. [61].

less important than the others. Lipid based systems can increase the internalization of the drugs, higher permeation, increase precorneal residence time, and sustained drug release with a minimum dosing frequency and decreased drug toxicity, which consequently promotes a higher improvement in ocular drug bioavailability and therapeutic success. Also, its nanometric size reduces the clearance by the eye's protective mechanisms due to its adhesive properties [67–69].

On another hand, the effectiveness of therapeutic proteins depends on their 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 drugs, the therapeutic effect is 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 their dosage form [58,59]. The use of lipid nanoparticles has several advantages including improvement of bioavailability and others [70, 71]:

- Biocompatibility and biodegradability
- Low toxicity
- Targeted and controlled 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 overcoming these limitations of therapeutic proteins. 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 like 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 [8].

Overall, lipid-based nanoparticles include liposomes, SLN and NLC fully disclosed in the following subsections.

5.1. Liposomes

Liposomes, an early version of lipid-based nanoparticles, are composed by lipids and fatty acids that are considered biocompatible and biodegradable owing to their natural occurrence in cell membranes. Additionally, their structure has early attracted the attention as a promising delivery system due to its flexibility, low immunogenicity, low toxicity, easy preparation, extended circulation time, and the ability to extend the shelf life of formulations [64,72].

Liposomes are small vesicles of spherical shape with particle sizes ranging from 100 nm to 1000 nm composed by at least two lipophilic layers [73]. These nanostructures are defined by the spontaneous assembly of phospholipids into a bilayer sphere, in which the hydrophilic head groups face the exterior aqueous environment, and the hydrocarbon chains assemble within the hydrophobic interior (Fig. 7). It was the first lipid nanostructure to be produced due to its self-production capacity owing to its amphiphilic character, since in aqueous solutions phospholipids impulsively form closed structures [73]. Their amphiphilic character also allows it to be a versatile drug delivery system for both hydrophobic and hydrophilic drugs [64,72]. Hydrophobic drugs are carried between the phospholipid layer, and hydrophilic in the aqueous core of the liposome [74].

Liposomal encapsulation of therapeutic drugs has been extensively used. In this sense, liposomes may be applied for protein delivery while keeping their structure and bioactivity. Water soluble proteins can be carried inside the aqueous core or can be attached to the lipid surface, while hydrophobic peptides or proteins are inserted into the inner hydrophobic center of the bilayer [75].

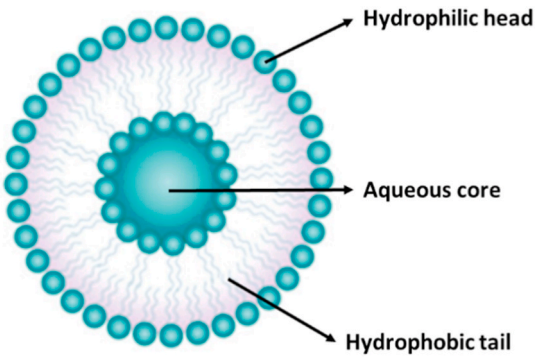

Fig. 7. Structure of a typical liposome. Adapted from Ref. [69].

Dawoud et al. loaded insulin into a chitosan-based spray in liposomes intended for wound healing by topical delivery. Different lipids were used and the effects of the cholesterol addition, method of preparation, and sonication were evaluated on the particle size and the entrapment efficiency. Liposomal insulin particle sizes ranged from 0.7 to 2.9 μm, depending on the use of cholesterol, since this lipid increased the diameter of the vesicle. The encapsulation efficiency of insulin varied between 37% and 84% depending on the preparation technique and the presence of sonication, which decreased the amount of the loaded drug. Finally, studying the behavior in Franz diffusion cells, the insulin dispersion, and the optimized liposomes formulation revealed a prolonged release of 6 h and up to 24 h, respectively. These findings revealed that topical insulin liposomal spray offered a protective method for insulin delivery [76].

Another formulated liposomal drug is the patented vaccine Epaxal®, a liposomal nanoparticle formulation of a protein antigen used as a hepatitis A vaccine, in which the viral envelope glycoproteins are intercalated in the phospholipid bilayer membrane. This structure facilitated the delivery of hepatitis A virus antigen to immunocompetent cells given the properties of the active fusion glycoproteins [77].

However, in some cases, protein delivery by liposomes has a rapid clearance by the mononuclear phagocyte system. One strategy to overcome this problem is the conjugation of the lipid surface with an inert polymer such as PEG. The steric impediment effect of PEG chains resulted in the increase of the hydrodynamic volume of the system, and the PEG capacity to avoid the immune response. However, it is important to avoid a high degree of conjugation with PEG as this results in a reduction in the melting temperature of the liposomes, which promotes their destabilization. So, PEGylation may just extend their circulation half-life from 30 min to 5 h [65,75].

Based on their size and number of bilayers, liposomes can be classified into three categories [64,74,78]:

- **Unilamellar vesicles (ULVs)** - vesicle has one bilayer membrane (a single phospholipid bilayer sphere enclosing the aqueous solution).
- **Oligolamellar vesicles (OLVs) –** vesicles with 2–5 bilayer membranes.
- **Multilamellar vesicles (MLVs) -** vesicles have five or more bilayer membranes in a structure like an onion.

Usually, different unilamellar vesicles encircle inside each other with successively smaller sizes, creating a multilamellar structure of concentric phospholipid spheres separated by layers of water [78].

The production method defines the type and size of liposomes produced and those influence drug encapsulation efficiency and circulation time [74]. Different production methods, as well as lipid compositions, can be used influencing the properties of these nanosystems, namely

surface charge and size. The surface charge of these nanostructures is usually determined by the charges of the lipid head groups, which can be positively or negatively charged or zwitterionic. This surface charge influences the interactions between particles and the adsorption of counterions, and thus the stability of the nanoparticles. Thus, uncharged particles or particles with low charge tend to aggregate over time, while more highly charged particles repel each other, preventing aggregation [79].

In addition, the type of bilayer components used in their preparation influence the 'stiffness' or 'fluidity' and the charge of the bilayer. An example of this is unsaturated phosphatidylcholine species from natural sources (phosphatidylcholine from eggs or soy) result in bilayers that are much more permeable and less stable, whereas saturated phospholipids with long acyl chains (e.g., dipalmitoylphosphatidylcholine) form a rigid and impermeable bilayer structure [64]. Other types of lipids are used in liposome preparations which allow modulating the nanostructure properties (Table 3).

However, liposomes revealed some drawbacks concerning stability problems over time such as easy sedimentation, aggregation, and coalescence that can shorten their shelf-life, resulting in loss of liposome-associated drugs, and changes in size. These issues lead to low reproducibility, reduced encapsulation efficiency, high polydispersity index, and unexpected and uncontrolled drug release during storage. In another hand, sometimes phospholipids undergo oxidation and hydrolysis-like reaction. In this sense, is crucial to control their stability during and after the production process [72,80]. Moreover, liposomes can suffer accumulation in liver and splenic macrophages, leading to splenomegaly and hepatotoxicity [81]. Therefore, due to the natural instability of liposomes, which limits their clinical use among other disadvantages, these nanostructures showed not to be robust enough for the delivery of proteins, so other lipid based nanocarriers were developed. Table 4 summarizes some more recent works.

5.2. Solid lipid nanoparticles

SLN were developed by Schwarz et al. (1994) and in parallel by Morel et al. (1998) [96,97]. 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 including:

- Allowing controlled drug release and targeting
- Increasing drug stability
- Allowing high drug payload
- Incorporating lipophilic and hydrophilic drugs
- Being composed of biocompatible lipids
- Large-scale production ability
- Use of Generally Recognized as Safe (GRAS) compounds and therefore low toxicity as carriers
- Improvement of drug stability and safety

They have been actively investigated for the delivery of drugs by different delivery routes [52,98,99]. The most frequently used excipients for SLN production are shown in Table 5.

The lipids compose the matrix of the SLN, and are solid at room and body temperature, usually with a melting point above 40 °C, used in a concentration ranging from 5 to 40%. Distinct types of lipids are used, ranging from triglycerides, partial glycerides, and fatty acids to steroids and waxes. This fact is one of the major advantages of SLNs, as they are made of physiologic materials decreasing the danger of acute and chronic toxicity of these nanoparticles. The choice of lipids relies on the solubility of the compound that is incorporated inside the matrix [66,100].

The emulsifier role in the formulation is to reduce the surface tension between the aqueous and lipid phases, thereby helping the stabilization of the system. Since they are amphiphilic molecules, they are placed in the interface of the system [52,98]. Several types of emulsifiers have been employed in SLN formulations, like as bile salts, ethoxylated alcohols, fatty acids, phospholipids, poloxamers, polyethylene glycols, polysorbates, polyvinyl alcohols, quaternary ammonium compounds, sorbitan esters, and tyloxapol, and it was discovered that a binary combination of emulsifiers helps to stabilize the systems more effectively and results in smaller nanoparticle sizes [66]. The choice of emulsifiers should take into consideration the hydrophilic-lipophilic balance (HLB) of the lipids employed in the formulation, as well as their concentration of the lipid phase and the administration route [52,98].

There are three incorporation models for the SLN that differ in the location and distribution of the loaded therapeutic protein within the lipid core as shown in Fig. 8.

- **SLN Type I/homogeneous matrix model –** In this model the drug is dispersed in the lipid core or as amorphous agglomerates. This type is usually produced by high pressure homogenization (HPH), either cold HPH or hot HPH with an optimized drug/lipid ratio. Usually, these nanoparticles show good controlled release properties.
- **SLN Type II/drug enriched shell model** - In this model it is obtained a drug-free lipid core surrounded by an outer shell containing the drug and the lipid. This model is used when a faster release of the encapsulated drug is desired.
- **SLN Type III/drug enriched core model** – In this model, the core of the nanoparticle is enriched with drug while the lipid is in the outer shell. This morphology is obtained when the drug concentration in the melted lipid mass is close to its saturation solubility and the lipid, when cooled, precipitates in the core before the lipid. This model is also suitable for drugs that require a prolonged drug release [71,101].

Nevertheless, the SLN has two main limitations related to its densely packed crystal structure: low loading capacity and drug expulsion during storage. Both lipophilic and hydrophilic active substances can be entrapped [66]. Drugs are mostly incorporated between the fatty acid

Table 3
Excipients used for liposome production. *Adapted from* Ref. [68].

Natural phospholipids	Synthetic phospholipids	Unsaturated
Phosphotidylcholine	1,2- Dilauroyl-sn-Glycero-3-Phosphocoline (DLPC)	1-Stearoyl-2-Linoleoyl-snGlycero-3-[Phospho-LSerine] (Sodium Salt)
Phosphotidylserine	1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (Sodium Salt) (DOPS)	Dioleaylphosphotidylcholine
Phosphotidylethanolamine	Dipalmitoylphosphotidylcholine	
Phosphotidylinositol	Distearoylphosphotidylcholine	
	Dipalmitoylphosphotidylseine	
	Dipalmitoylphosphotidylglycerol	
Others		
Sphingolipids - Shingomyellin		
Glycosphingolipids - Gangliosides		
Steroids – Cholesterol		
Polymeric material - **Lipids** conjugated to diene, methacrylate & thiol group		
Charge-inducing lipids - **Diotadecyldimethyl** ammonium bromide/chloride (DODAB/C); Dioleoyl trimethylammonium propane (DOTAP)		

Table 4
Applications of lipid nanoparticles.

Nanocarrier	Name	Nanocarriermatrix	Preparation method	Load	Application	Ref.
Liposome	Mosquirix™ vaccine RTS, S/AS01	1,2-dioleoyl-sn-phosphatidylcholine [DOPC] and cholesterol-based	–	Circumsporozoite protein, chemical adjuvant (AS01E) and a viral surface antigen of the hepatitis B virus (HBsAg)	Malaria - *Plasmodium falciparum* and in a lesser extent Hepatitis B	[82]
	Cationic liposomes-protein conjugate complex (GBS67-CpGODN + L)	1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol and dimethyldioctadecylammonium bromide (DDA)	Microfluidics	Group B Streptococcus GBS67 protein antigen with the CpG oligodeoxynucleotides (CpGODN)	Hepatitis B	[83]
	Lipo-AE vaccine	Phosphatidylserine	Sonication	*Mycobacterium tuberculosis* antigens (Ag85B and ESAT-6)	*Mycobacterium tuberculosis*	[84]
	DDA/TDB/CHOL liposomes	DDA, trehalose-6,6′-dibehenate (TDB) and cholesterol	Thin film method	*Mycobacterium tuberculosis* fusion protein (HspX, PPE44, and EsxV antigens)	*Mycobacterium tuberculosis*	[85]
	Liposome-based vaccine	Alpha galactosylceramide	Film extrusion method	Palmitoylated synthetic long peptides	Dendritic cells	[86]
	Insulin-Loaded Liposomes	Phosphatidylcholine and cholesterol	Dry thin film hydration method	Insulin	Wound Healing	[87]
	Annexin A5-associated Liposomes	Phosphatidylserine and phosphatidylethanolamine	Thin film hydration method	Ranibizumab and Bevacizumab	Antibodies delivery to the retina	[88]
SLN	Cyclosporine A -loaded lipid nanoparticles	Lipocire DM and Pluronic F-127	Hot homogenization method	Cyclosporine A	Skin-related diseases	[89]
	Erythropoietin -loaded SLN	Glycerin monostearate, span®80/ span®60 and tween®80	Double-emulsion solvent evaporation method	Erythropoietin	Neurodegenerative disorders (Alzheimer's disease)	[90]
	Coenzyme Q10 - SLN	Compritol 888 ATO, Poloxamer 188 and Tween 80	High shear homogenization method	Coenzyme Q10	Antioxidants dermal delivery	[91]
	PEG-coated lipid nanoparticles	Miglyol® 812 and tripalmitin	Double emulsion-solvent emulsification method	Peptide salmon calcitonin	Oral administration of peptide drugs	[92]
	Tobramycin-SLN	Stearic acid, Epikuron 200 as and sodium taurocholate	Microemulsion	Tobramycin	Intraocular tobramycin delivery	[93]
NLC	Coenzyme Q10 - NLC	Stearic acid, oleic acid, isopropyl myristate and isopropyl palmitate	High shear homogenization method	Coenzyme Q10	Antioxidants dermal delivery	[94]
	Chitosan coated NLC	Precirol ATO5, Dynasan 114, Miglyol, Tween 80 and Poloxamer 188	Sonication	Model proteins	Brain delivery of proteins by intranasal administration	[76]
	Ovalbumin-NLC	Suppocire NB™, Super refined Soybean oil, lecithin, glycerides and Lipoid S75™	Ultrasonication	Antigen ovalbumin	Development of vaccine formulations	[95]

chains, lipid layers, or in the amorphous clusters of the crystal imperfections. SLN usually crystallizes in a perfect lattice, especially 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 [102]. 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 [103]. Additionally, SLN dispersion may suffer gelation phenomenon once its viscosity increases during the cooling process which results in a viscous gel and consequently leads to an increase in particle size and particle agglomeration [66].

In a study where bovine serum albumin (BSA) was used as a model protein for the encapsulation into a matrix modified by incorporation of lecithin into the lipid matrix and 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 [104]. In a different study, using SLN for the encapsulation of lysozyme, the method used produced formulations with reduced concentration of protein and low encapsulation efficiency, which considering the excessive 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 [105]. In Table 4 it is summarized more works focusing on SLN for protein delivery.

5.3. 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. The structural differences between SLN and NLS are shown in Fig. 9. Therefore, the 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 ranges from 1.5% to 5% (w/v). The excipients employed in the production of NLC are the ones used for SLN plus a liquid lipid [71, 98,106] – Table 5. The liquid lipid could be fatty alcohols, medium-chain triglycerides, paraffin oil, and squalene. Moreover, fatty acids, such as oleic, linoleic, and decanoic acid may be used since their properties as penetration enhancers [100].

Table 5
Excipients for SLN and NLC production. *Adapted from* Ref. [79].

Excipients	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 and 70 °C and HLB between 1 and 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 several 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 and 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 non-irritant, 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

Same as the SLN, according to the production method, and the composition of the lipid mixture, there are three types of NLC (Fig. 10).

- **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** – In this type, it is used solid special lipids as hydroxyoctacosanyl hydroxy stearate or isopropyl 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 type 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

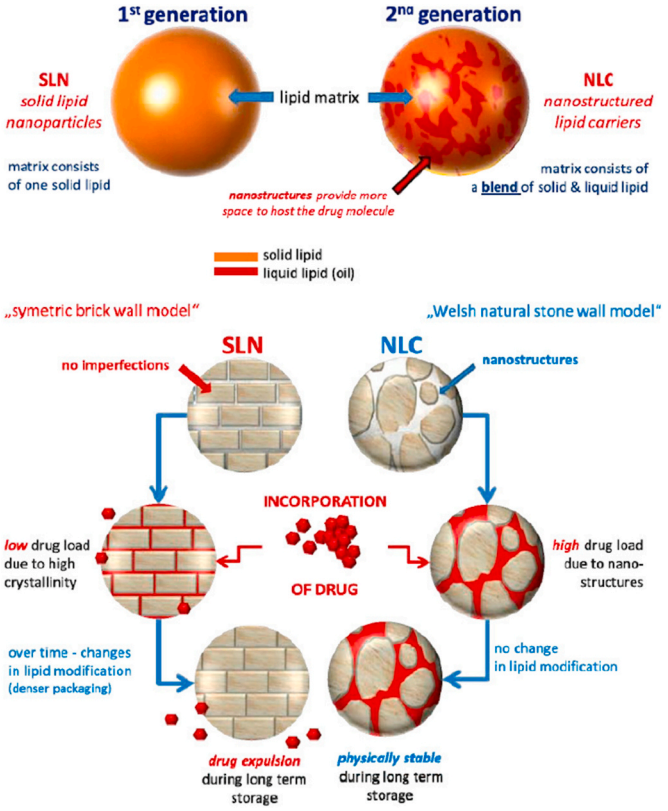

Fig. 9. Structural differences between SLN and NLC. *Adapted from* Ref. [107].

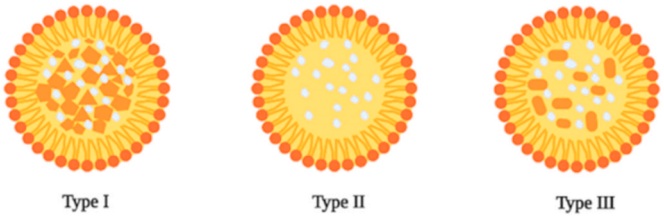

Fig. 10. Types of NLC. Reprinted from Ref. [81].

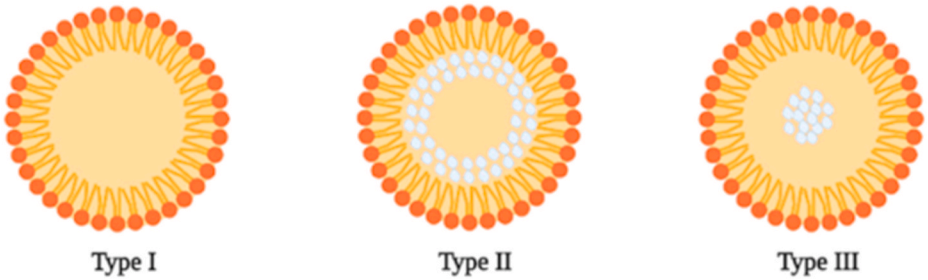

Fig. 8. Types of SLN. *Reprinted from* Ref. [81].

prolonged release because the compartments are surrounded by solid lipids [71,101].

In the last years, the number of papers on NLC formulations increased considerably and it has been emerging as an ideal drug delivery system for the pharmaceutical market. However, studies addressing the delivery of therapeutic proteins by NLC are scarce, due to formulation production challenges.

In a recent study, it was developed coenzyme Q10-loaded (co-Q10) NLC by the high shear homogenization method, obtaining spherical nanoparticles with an average particle size of 180–350 nm, a PdI below 0.5, zeta potential below −0.3 mV and an encapsulation efficiency between 83 and 88% [108]. 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.

Another study developed by Rocha et al. proposed the use of nanostructured systems to enhance the antimicrobial activity of antibiotics, namely the polypeptide bactericidal antibiotic Polymyxin B by functionalization. NLC loading dexamethasone acetate and its surface were modified by polymyxin B sulfate were developed intended to increase the antimicrobial activity against *P. aeruginosa*. NLC was obtained by high-pressure homogenization and coated with polymyxin B. The produced formulation revealed good stability and physicochemical characteristics and this new carrier platform showed an enhanced polymixin B antimicrobial activity 2- to 3-fold against *P. aeruginosa* revealing that this peptide conjugation strategy may be a new successful treatment against gram-negative bacterial infections [66].

In the literature, there is not much more description of NLC for the encapsulation of therapeutic proteins **(Table 4).** This is mainly because production methods usually use 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 techniques are the preferred methods [102,109]. Since NLC are obtained 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 change the paradigm of therapeutic proteins, allowing their administration by different delivery routes and optimize their use as therapeutics.

6. Production methods of therapeutic proteins-loaded lipid nanoparticles

6.1. Liposomes production

Liposome production methods, in general, involve the following steps: the extraction of lipids from the organic solvent; their dispersion in an aqueous solvent or buffer; the purification of the liposomes formed; and the analysis of the final product [64,110]. During preparation, the types and amounts of phospholipids, the ionic and polarity properties of the aqueous medium, and the techniques used are crucial factors that determine the final structure of the liposome. The encapsulation of therapeutic proteins occurs passively during liposome formation or actively after liposome preparation. Among the main passive loading techniques, the mechanical dispersion methods (sonication, extrusion, freeze-thaw, thin film hydration, and microfluidization), solvent dispersion method and removal of detergent or non-encapsulated material are the main ones. The isolation of liposomal vesicles is based on the principles of dialysis, adsorption, gel permeation chromatography, and dilution [64,111].

6.1.1. Sonication

Sonication is the most frequently used technique to prepare small ULVs. For the preparation of MLVs sonication occurs in a bath-type sonicator, or a probe sonicator under a passive atmosphere. The protein solution is added to the surfactant and cholesterol solution, while the mixture is sonicated at the surfactant transition temperature for minutes. However, this method presents low encapsulation efficiency, and may enhance the degradation of phospholipids and drugs to be encapsulated [64,112].

6.1.2. Extrusion

The extrusion method involves passing the suspension of MUVs through a membrane filter of defined pore size to form small ULVs, being more suitable for labile materials, like proteins, than sonication [113]. The application of high pressure helps the extrusion of the vesicles through the small polycarbonate pores transforming large vesicles into small ones due to the passage through the pores. The properties of the liposomes formed namely, the average size and polydispersity, depend on the applied pressure, the number of cycles, and the pore size of the filters used. This is a simple, fast, and reproducible method that gives rise to homogeneous size distributions. Thus, the main disadvantages of this method are blockage of the pores, possible loss of product, and the working volumes are relatively small [112,114,115].

6.1.3. Thin film hydration

In this method, the surfactants and lipid molecules are solubilized in an organic solvent or a mixture of volatile organic solvents. Then, by reducing the pressure the solvent is evaporated, leaving a thin film of lipids. Then, a large volume of protein aqueous solution is added slowly to the film on the inner surface of the container at a temperature above the transition temperature of the lipid used. The volume of the aqueous solution used and this hydration step, where one phase interacts more with water than the other, influences the properties of the liposomes. High water volumes lead to the formation of MLVs, while the speed of hydration determines the efficiency of protein encapsulation, so the slower the hydration speed, the higher the encapsulation efficiency [72,112].

6.1.4. Freeze-thaw

In this technique the small ULVs are rapidly frozen and slowly thawed. The first step consists of forming liposomes by thin film hydration technique, followed by freezing at −196 °C for 5 min together with the therapeutic protein. This is followed by a rapid transfer to a water bath at surfactant transition temperature for 5 min. This cycle is repeated 2–4 times so the drug is efficiently enclosed in the vesicles during the cycles. The formation of unilamellar vesicles results from the fusion of small ULVs throughout the freezing and thawing processes, and these are critical steps for drug encapsulation efficiency and liposome stability which protects the protein [64,72,112].

6.1.5. Microfluidization

The microfluidizer is a high-pressure equipment that converts high fluid pressures to intense shear forces, employing a pressure current applied through a thin opening that generates a flow inside the microfluidizer chamber. Liposomes are formed by converting high pressure into a combination of high shear and impact forces, high energy dissipation as well as hydrodynamic cavitation. The lipids are dissolved in an alcoholic solvent and passed through the central channel, while a protein aqueous solution is added to the two adjacent channels. Lipid and aqueous fluxes are concentrated at the point of intersection, and flow velocities will determine the flux concentrations at the point of intersection. Thus, the size and distribution of the nanoparticles are controlled by varying the lipid concentrations and flow conditions. This technique allows to produce ULVs with the desired sizes, with low variability, and in a reproducible way. The major disadvantage is the application of high pressures during the process that could damage the

therapeutic protein [112,115].

6.1.6. Other methods

Concerning solvent dispersion methods such as ether injection and ethanol injection techniques, lipids dissolved in organic solvent or ethanol are injected into an aqueous solvent or buffer solution containing materials to be encapsulated under reduced pressure. However, the techniques by which the vesicles are formed result in very heterogeneous vesicles, which can result in the inactivation of therapeutic proteins [113].

6.2. Solid lipid nanoparticles and nanostructured lipid carriers production

Both SLN and NLC are produced using the same methods, which are briefly shown in Fig. 11 and further detailed in this section [98,116]. From the different 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 delivery 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, those methods must be optimized for the encapsulation of therapeutic proteins, which are shear and temperature-sensitive compounds, and therefore require methods that avoid or mitigate those stresses [104].

6.2.1. High pressure homogenization

HPH emerged as a reliable, well-established, and widely used technique to produce lipid nanoparticles. Some of the advantages of this method include the possibility of large-scale production, avoidance of organic solvents and attaining particles with an average size on the submicron region. For this technique homogenizers are used that push the liquid with high pressure, between 100 and 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

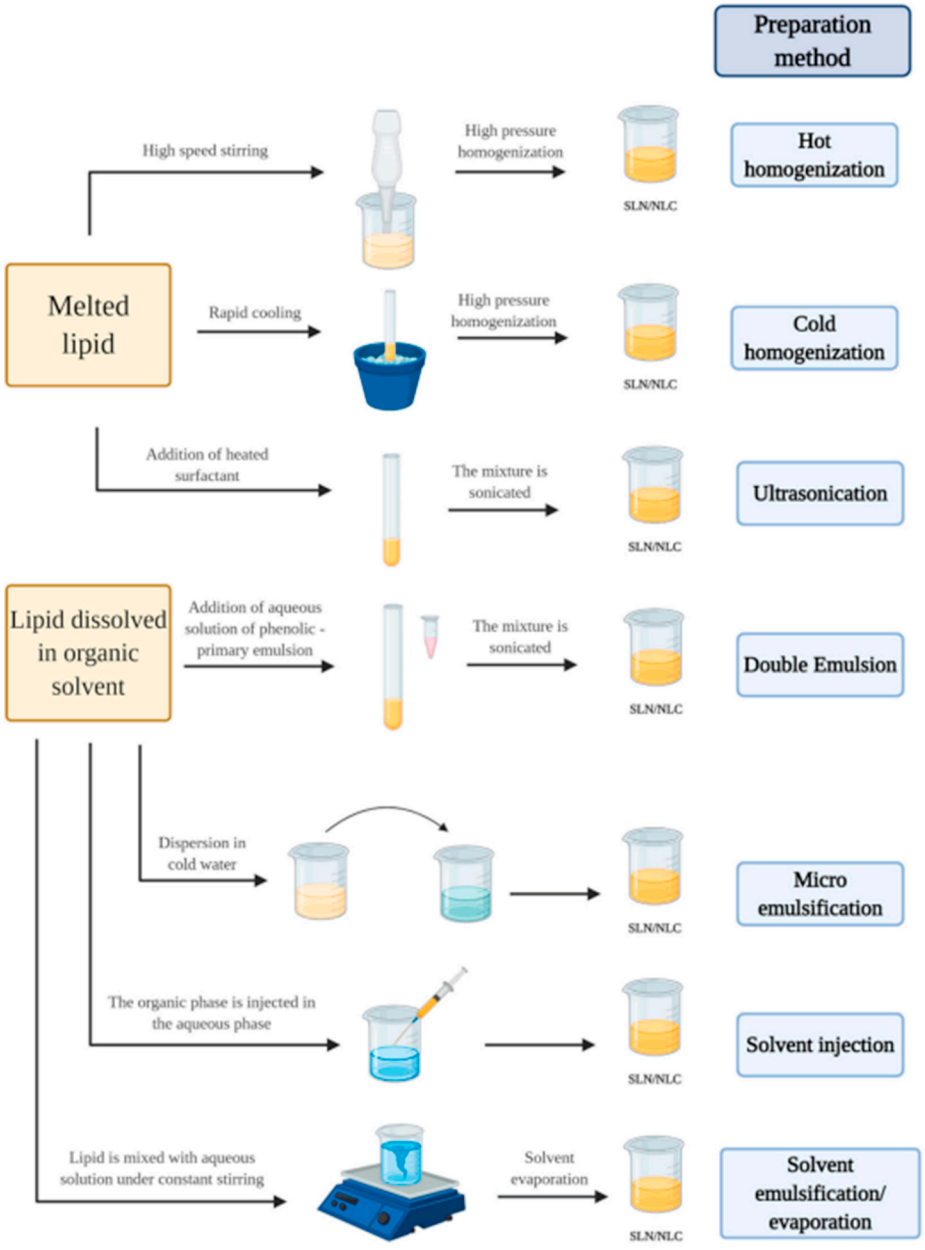

Fig. 11. Methods for SLN and NLC production. *Reprinted from* Ref. [81].

forces inherent to the process reduces 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 (Fig. 12). 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 5 °C above the lipid melting point [52,98]. The hot HPH is less adequate for the encapsulation of therapeutic proteins due to the elevated 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 managed 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 protein remained intact during all the harsh conditions of the procedure, but the encapsulation efficiency was only about 59%, because 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 [8,105]. On the other hand, results using BSA showed the temperature and pressure conditions of HPH strongly influenced the protein structure. Other studies using human insulin and cyclosporine A showed both proteins maintained their structures, with cyclosporine A showing an incorporation efficiency above 90% [8].

6.2.1.1. Hot high pressure homogenization. In this technique, the entire process is performed at temperatures above the lipid melting point, and under high shear stirring a pre-emulsion is prepared. The lipid melt, and the aqueous emulsifier are mixed, both at the same temperature. After that, the formed pre-emulsion is homogenized by HPH. Hot 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 enough to produce SLN and NLC with an average particle size ranging from 250 to 300 nm, when the pre-emulsion concentration is between 5 and 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 stage, an emulsion is obtained due to the physic state of the lipid. The last step is cooling the sample at room temperature or lower, leading to lipid crystallization and formation of the nanoparticles [52,98].

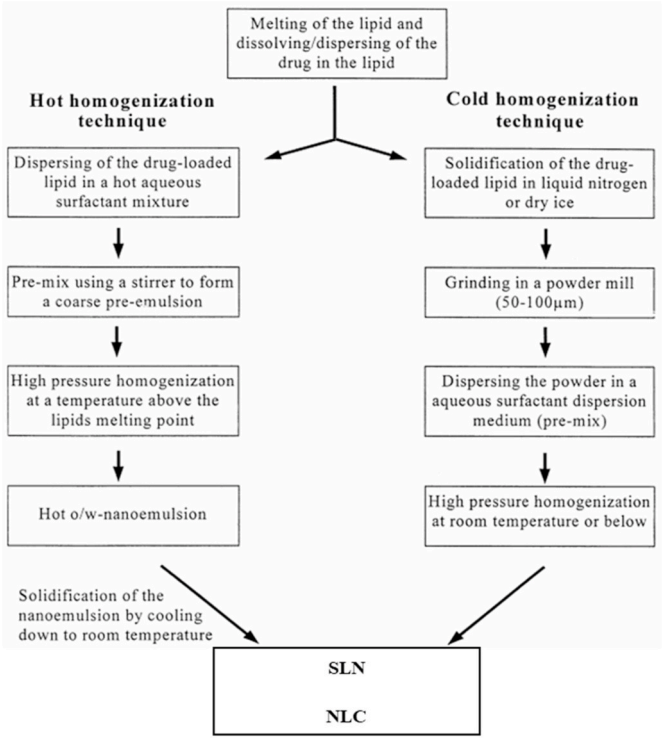

Fig. 12. Schematic representation of Hot and Cold Homogenization for SLN and NLC production. *Adapted with permission from* Ref. [52].

According to the literature, this technique can be used for some heat-sensitive- compounds because the time of exposure to elevated temperatures is short. Even so, the temperature employed is one of the limitations of this technique since, as mentioned, it is unsuitable for overly sensitive compounds and hydrophilic compounds that in elevated temperatures can partition from the lipid phase to the aqueous phase [52,98]. Nevertheless, there is no relevant works 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 structure of the proteins and compromise its therapeutic effect.

6.2.1.2. Cold high pressure homogenization. This technique was developed to overcome the limitation of hot HPH: degradation of the loaded bioactive due to elevated 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. 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 drug. Then, the obtained solid 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 main 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 those formed using the hot HPH [52,98].

6.2.2. Emulsification methods

The emulsification methods are the best to load therapeutic proteins into lipid nanoparticles, due to the avoidance of elevated temperature and shearing stress. In this section different emulsification methods are addressed.

6.2.2.1. Ultrasonication. Ultrasonication is a dispersing technique, on which the lipid nanoparticles are obtained by dispersing the melted lipid phase in the aqueous phase with the surfactant. It allows the cleavage of large particles into smaller ones, by providing energy, usually above 20 kHz of ultrasonic rates/frequencies for homogenization [71,117]. In the first step of this technique, the lipid is melted, about 5–10 °C 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 last step, the emulsion is cooled at a temperature under the solidification temperature of the lipid, with the formation of a nanoparticle dispersion [71]. 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 [98].

In a previous work, 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 times 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 about 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 on size and zeta potential, applying the same parameters using different lipids. The resulting SLN were after evaluated to determine the short-term stability in aqueous dispersions. The mean particle sizes of SLNs made of SA, cetyl palmitate, Precirol, Dynasan118 and Compritol were about 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 [118].

6.2.2.2. Double emulsification. 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 large [71]. 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 [119].

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, and 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 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 [120].

6.2.2.3. Microemulsification. The first step of this homogenization technique consists of 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 microemulsion the system is dispersed in chilly water with a temperature ranging from 2 to 10 °C, 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 [22]. 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 [7,98].

The first attempt to encapsulate peptide drugs in SLN was carried by Morel et al. (1994) using this technique for the encapsulation of triptorelin and thymopentin as model peptides [82,83]. The encapsulation efficiency was low in both cases and equivalent results were observed for the encapsulation of cyclosporine A [8].

6.2.3. Solvent evaporation

In this method, 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 [52,84]. This approach also avoids temperature and high-energy sources, and it results in particles with a narrow size distribution [98,85].

Overall, this is a widespread method in the preparation of nanoparticles, including SLN. For hydrophilic compounds, including proteins, associating the double emulsion technique to this method was demonstrated to improve their encapsulation efficiency. Thus, a big part of the studies with protein 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 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 nanoparticles (GCSLN) were 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 a 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 12 h [86].

6.2.4. Solvent injection

In this method a transitional o/w emulsion is prepared 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. First, 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 [71]. This method uses mild organic solvents, avoids several critical as elevated temperatures, high pressures, and high emulsifier concentrations, and has emerged as an efficient, versatile, and easy to implement technique [98,87].

The solvent injection method was firstly used to produce lipid nanoparticles by Schubert et al. (2003). The results from this study showed that acetone, ethanol, isopropanol, and methanol are suitable solvents for the preparation of lipid nanoparticles, which was not verified with ethyl acetate that was not able to successfully produce the nanoparticles. The particle sizes obtained were 80–300 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 when compared to the bulk lipid [87].

7. 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 delivery of therapeutic proteins allows a new range of opportunities. The use of lipid nanoparticles has the potential to overcome the delivery

challenges of therapeutic proteins, allowing their delivery by different administrations routes. Thus, therapeutic proteins may be loaded into liposomes, SLN and NLC. However, the use of the NLC for the encapsulation of therapeutic proteins is not well established yet, and it urges the need to optimize production methods that could not compromise the protein structure during the encapsulation process. It is foreseen that the use of lipid nanoparticles to deliver therapeutic proteins will keep on growing the upcoming years, since several challenges still need to be properly addressed. This review disclosures those challenges and points out some paths to follow.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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