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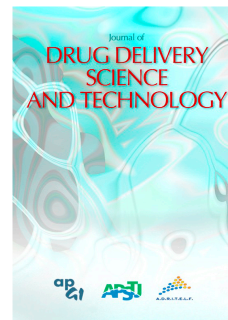

An insight on lipid nanoparticles for therapeutic proteins delivery

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PII: S1773-2247(22)00750-X

DOI: https://doi.org/10.1016/j.jddst.2022.103839

Reference: JDDST 103839

To appear in: *Journal of Drug Delivery Science and Technology*

Received Date: 6 July 2022

Revised Date: 14 September 2022

Accepted Date: 23 September 2022

Please cite this article as: Clá. Viegas, F. Seck, P. Fonte, An insight on lipid nanoparticles for therapeutic proteins delivery, *Journal of Drug Delivery Science and Technology* (2022), doi: https://doi.org/10.1016/j.jddst.2022.103839.

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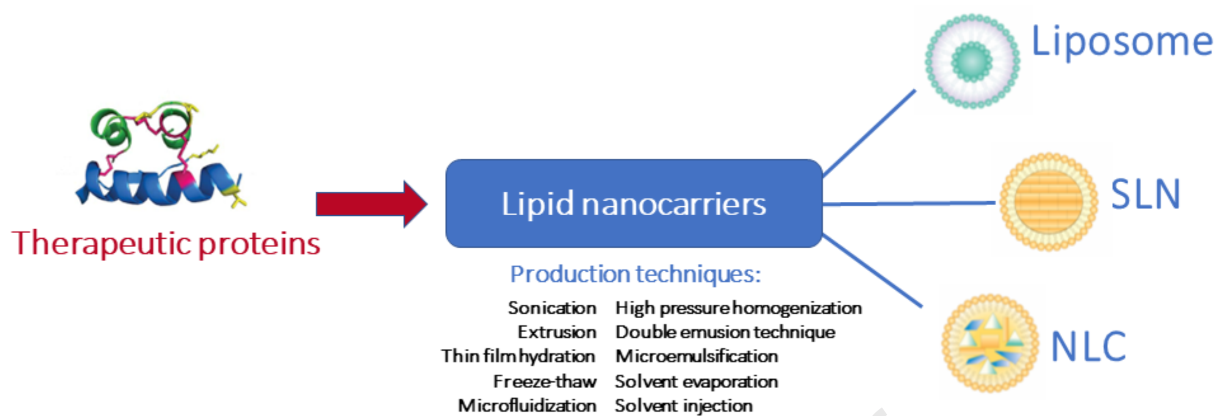

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1 # An insight on lipid nanoparticles for therapeutic proteins delivery

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An insight on lipid nanoparticles for therapeutic proteins delivery

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.

Keywords: Delivery system; Encapsulation; Liposome; Nanostructured lipid carrier; Solid lipid nanoparticle; Therapeutic protein.

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58 **List of abbreviations and acronyms:**

59

60 ADME - Absorption, distribution, metabolism, and excretion

61 Ala - Alanine

62 BSA – Bovine serum albumin

63 CNT - Carbon nanotubes

64 DNA - Deoxyribonucleic acid

65 EE – Encapsulation efficiency

66 GCSLN - Gel core solid lipid nanoparticles

67 GRAS - Generally recognized as safe

68 HLB - Hydrophilic-lipophilic balance

69 HPH – Hot pressure homogenization

70 LC - Loading capacity

71 MLV- Multilamellar vesicle

72 NLC – Nanostructured lipid carriers

73 OLV - Oligolamellar vesicle

74 PdI - Polydispersity index

75 PEG – Polyethylene glycol

76 pI – Isoelectric point

77 SLN – Solid lipid nanoparticles

78 The - Threonine

79 ULV - Unilamellar vesicle

80 Val - Valine

81 VB12 - Vitamin B12

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131 **1. Introduction**

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

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

151 In the last years, lipid nanoparticles have been studied as drug delivery systems, as
152 an attempt to overcome the problems and improve the characteristics of therapeutic
153 proteins, protecting it from degradation *in vivo*, allowing a controlled release, modifying
154 biodistribution, and enhancing targeted delivery, solubility, and bioavailability. The use
155 of lipids in the development of delivery systems started with phospholipid vesicles named
156 as "liposomes" in 1965 by Prof. A.D. Bangham. Liposomes are spherical vesicular
157 systems, composed of one or multiple phospholipid bilayers entrapping an aqueous phase,
158 firstly introduced in the cosmetic market in 1986, and after in pharmaceutical products at
159 the end of the 1980s [5–7]. A few years later, it was developed a new generation of lipid
160 nanoparticles, the solid lipid nanoparticles (SLN), and even later the nanostructured lipid
161 carrier (NLC) made. The SLN are composed of a solid lipid matrix of biodegradable and
162 biocompatible lipid or blend of lipids. The NLC were developed to overcome the
163 disadvantages of the SLN related to their perfect crystallization matrix structure, which
164 is responsible for the SLN low loading capacity and undesired expulsion of the

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165 encapsulated drug during storage. By presenting a matrix composed of two different
166 lipids, the NLC matrix presents imperfections that increase their loading capacity, better
167 accommodating the encapsulated drug, avoiding drug expulsion during storage, and
168 allowing better sustained released properties compared to SLN [5–7]. Both SLN and NLC
169 use similar production methods which are very well established but usually not suited for
170 the encapsulation of therapeutic proteins, because they often apply temperature and/or
171 high pressures that can damage the structure of the protein and compromise its bioactivity
172 [1,8].

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

177

178 **2. Therapeutic proteins**

179 Jöns Jakob Berzelius first used the term "protein" in 1838. These molecules were
180 identified in the 18th century as having specific biological properties, namely the ability
181 to coagulate when treated with heat or acid [9]. Currently, there are over 250 proteins
182 used clinically for different purposes from prophylaxis as is the case of some vaccines, to
183 clinical treatment of metabolic diseases or even cancer [10,11]. Therapeutic proteins are,
184 by definition, macromolecular drugs produced by biotechnology, using live organisms
185 and their active compounds [12]. The best example in the production and use of
186 therapeutic proteins is the history of insulin in the treatment of diabetes *mellitus*. Insulin
187 is an anabolic heterodimer composed of two chains, the A-chain with 21 residues and the
188 B-chain with 30 residues, both linked by two disulphide bonds and an additional
189 intrachain disulphide bond present in the A-chain, as shown in **Figure 1** [13]. In 1922
190 insulin was first purified from bovine and porcine pancreas, and used for treatment of
191 diabetic patients, emerging as a life-saving treatment [14]. Nonetheless, with the
192 widespread use of this protein, some problems became known: the limited availability of
193 animal pancreas for purification of insulin which would not be sufficient for the daily
194 treatments of patients, the cost associated with the process, and the immunological
195 reactions developed by some patients. To solve such problems and take advantage of the
196 advances in bioengineering, the human insulin gene was isolated and *Escherichia coli*
197 was engineered to express the human insulin, using recombinant deoxyribonucleic acid

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198 (DNA) technology. By growing enormous quantities of this bacteria, the large-scale
199 production of human insulin was accomplished and, in 1982, recombinant insulin was
200 approved by the Food and Drug Administration (FDA), representing one of the biggest
201 achievements of modern healthcare science [14].
202 Recombinant DNA technology established on an industrial scale has dramatically
203 escalated the number of biotechnology drugs approved and under investigation.
204 According to numbers from 2018, therapeutic proteins alone, excluding peptides and
205 genetic-based ones, corresponded to 199 entities in the United States of America (USA),
206 Europe, and Canada. Moreover, according to a study led by the Business
207 Communications Company (BCC) Research, the global market for bioengineered protein
208 drugs in 2016 was evaluated at $172.5 billion and it is expected to have reached $228.4
209 billion by 2021. In terms of the annual growth rate, in 2016 was $39.8 billion and it is
210 expected to have reached $40.2 billion in 2021, which is about 10% of the ethical
211 pharmaceutical market [15,16]. The increasing number of protein therapies that have been
212 used for a wide range of applications include hormones, enzymes, clotting factors,
213 antibodies and may be classified according to their pharmacologic activity or grouped
214 into molecular types as shown in **Table 1** [12,14,17–20].
215 Therapeutic proteins have several advantages over synthetic drugs. Firstly, they
216 present high specificity and cover a wide range of functions that cannot be mimicked by
217 chemical compounds. Since their biological action is extremely specific, the risk of
218 interfering with biological processes and causing adverse reactions is significantly lower.
219 In general, they are also very well tolerated because the body naturally produces many of
220 the proteins that are used as therapeutics. From a financial perspective, they are also more
221 appealing when compared to synthetic drugs for two particularly important reasons. The
222 first reason is related to the fact that the clinical development and approval time of protein
223 drugs is more than one year faster than for synthetic drugs. The results from a 2003 study
224 showed that the average clinical development and approval time for 33 therapeutic
225 proteins approved between 1980 and 2002 was more than 1 year faster than for 294 small-
226 molecule drugs approved during the same period [21]. A more recent study of clinical
227 drug development success rates from 2021 analysed 6151 successful phase transitions
228 during the 2011–2020 period, concluding that it took in average 10,3 years for a
229 therapeutic protein to reach the market, including 2,3 years at Phase I, 3,6 years at Phase
230 II, 3,3 years at Phase III, and 1,3 years at the regulatory stage [22]. In **Figure 2** it is
231 represented the duration of the phases of development for therapeutic proteins by disease,

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232 and in **Figure 3** for synthetic drugs. Moreover, due to their singularity in terms of form
233 and function, companies can obtain far-reaching patent protection [14].
234 Nonetheless, as shown in **Table 2**, despite all the advantages, the administration of
235 protein drugs still represents a challenge, due to their immunogenicity problems, poor
236 bioavailability due to their physicochemical instability and consequent fast degradation
237 in serum, production challenges and difficulty to permeate membranes as the
238 gastrointestinal epithelium. Since therapeutic proteins suffer rapid degradation in serum
239 and fast elimination, they are usually administered parenterally in high and repeated doses
240 to maintain it in therapeutic concentrations for the desired time, which is painful and not
241 well tolerated by patients, decreasing patient compliance to treatment. Furthermore, due
242 to their short residence period in blood before suffering renal clearance and enzymatic
243 degradation, it urges the need to administer high doses to reach therapeutic concentrations
244 for the desired period. This administration profile creates a variable concentration of the
245 therapeutic protein preceded by a high initial peak that leads to side effects [17,23]. To
246 diminish those side effects and address their narrow therapeutic ranges, several
247 approaches have been developed and evaluated to extend the therapeutical proteins half-
248 life in circulation. By extending the proteins half-life, both problems mentioned would be
249 addressed, maintaining the therapeutical concentrations with lower doses [17,23,24].
250 If it is true that therapeutic protein is one the fastest growing class of drug
251 molecules, is also true that developing strategies to overcome the obstacles imposed by
252 its administration problems are crucial to increase the number of formulations reaching
253 the pharmaceutical market [11,17].
254

255 3. Delivery challenges of therapeutic proteins

256 As previously mentioned, therapeutic proteins have delivery challenges that
257 compromise their therapeutic effect and limits delivery routes. From those, their
258 immunogenicity, short half-life, isoelectric point (pI), and modification of the protein
259 charge, structural stability and membrane permeation, and glycosylation profile are the
260 most impactful and are discussed in this section.
261

262 *3.1. Immunogenicity*

263 The development of therapeutic proteins was followed by the expectation that the
264 same as the "self" derived proteins, they would avoid immunogenicity. Unfortunately,

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265 this idea has been proven to be flawed, with several examples of recombinant proteins
266 that stimulate host immune responses, originating anti-therapeutic antibody response. The
267 generation of these anti-therapeutic antibodies involves stimulation of multiple
268 components of the immune system, both adaptative and non-adaptative immune
269 responses, which means that immunogenicity of protein therapeutics cannot be imputed
270 to a single factor. This is a serious and concerning problem, since these responses can
271 have a neutralizing effect on the protein, reducing the protein half-life or triggering
272 allergic reactions if the therapeutical is non-endogenous alike. But if the protein drug has
273 antigenic similarities with an endogenous protein, then a neutralizing antibody response
274 can cross-react with the endogenous protein, resulting in scenarios of morbidity and
275 mortality. Moreover, the immunogenicity of protein therapeutics is remarkably hard to
276 predict before clinical trials because the traditional animal models used for synthetic
277 drugs are of limited application for therapeutic proteins [25].

278 There is also a relationship between aggregated proteins and enhanced
279 immunogenicity, with studies showing this correlation in a variety of models [25]. Protein
280 aggregation is defined as the self-association of monomers either in their native or
281 partially unfolded forms, a process that can occur during the life of a therapeutic protein
282 induced by a wide range of factors like temperature, mechanical stress, freezing, and
283 thawing [26–30]. According to a study developed by Braun *et al.* (1997), the IFN-alpha
284 protein aggregates (IFN-alpha-IFN-alpha and human serum albumin (HSA)-IFN-alpha
285 aggregates) presented considerable higher immunogenicity than the IFN-alpha
286 monomers. The results from a study in 2011, also showed augmented immunogenicity of
287 aggregated rhIFNβ-1a in transgenic mice [31].

288

289 ***3.2. Short half-life***

290 Pharmacokinetics is, by definition, the study of the movement of xenobiotics
291 (drugs/compounds/chemical entities) within the body after administration, being affected
292 by four distinct, yet interrelated processes: absorption, distribution, metabolism, and
293 excretion (ADME) [32]. The efficacy of therapeutical proteins is significantly affected
294 by their pharmacokinetic properties as their plasma half-life [33].

295 Since most of the activity of the endogenous protein resembles hormones activity,
296 they frequently present fast serum elimination, which is desirable from the hormonal
297 regulation point of view. Nonetheless, therapeutic proteins are completely metabolized

298 through the same catabolic pathways as endogenous or dietary proteins, which leads to
299 also fast clearance, or nonmetabolic elimination pathways as renal or biliary excretion.
300 Depending on the protein size, renal filtration can be determinant for the protein
301 half-life in serum. Two main factors affect kidney filtration: protein size and
302 hydrophobicity. The kidney filtration cut off size for a peptide is < 70 kilodalton (kDa)
303 which means that peptides smaller than that will easily get cleared by the kidneys, which
304 also means that as the hydrodynamic radius of the protein increases, the renal
305 clearance decreases [32,34,35]. Yet, there is a wide diversity of therapeutic proteins
306 including monoclonal antibodies, enzymes, hormones, growth factors, and cytokines,
307 each one with specific average molecular weights, making this class very heterogeneous
308 in terms of the range of molecular weights.
309 Deamidation occurs when the amide groups of asparaginyl or glutaminyl residues
310 are hydrolysed to a free carboxylic acid because of susceptibility to extreme pH
311 conditions. This is also responsible for the short half-life of therapeutic proteins.
312 Yan *et al.* (2018) studied the impact of the deamidation rate of asparagine in the
313 protein structural features. Different stress conditions were employed, using extreme pH
314 (8.5) and high-temperature stress (37 °C) to identify the asparagine sites sensitive to
315 deamidation in IgG mAbs [36]. The results showed that the difference in asparagine
316 deamidation rate could be due to structure conformation, structure flexibility, and solvent
317 accessibility [37].
318

319 ### *3.3. Isoelectric point and protein charge*

320 Globular proteins are actively adsorbed to hydrophobic and hydrophilic interfaces
321 as production tanks, glass vials, or processing components, which significantly influence
322 their pharmacokinetics and biodistribution, leading in some cases to aggregation of the
323 therapeutic protein and eventually to a decrease in concentration. The pI, which is the pH
324 of a solution at which the protein maintains zero net charge has a considerable influence
325 on the adsorption of proteins to hydrophilic and charged surfaces. According to a
326 therapeutic protein local physiological environment, the overall charge of the protein can
327 vary which means that according to the strength of the interaction, the therapeutic proteins
328 may be adsorbed [35,37].
329 A study on the characterization of protein adsorption onto nanoparticles,
330 highlighted the impact of isoelectric interactions on globular proteins Lyz and ß-Lg onto

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331 negatively charged nanoparticles. In both cases, it was verified that for low pH values,
332 the competition between the attractive protein-surface and the repulsive protein-protein
333 interactions limited the adsorption to one monolayer of the protein molecules. For pH
334 values closer to pI the protein-protein interactions were less relevant which extended the
335 adsorption significantly above one monolayer [38].
336

337 ### *3.4. Structural stability and membrane permeation*

338 Therapeutic proteins present high susceptibility to suffer chemical and physical
339 degradation. Physical instability refers to events that lead to conformational changes in
340 the protein structure that includes protein unfolding, aggregation, precipitation, and
341 adsorption to the surface. Chemical instability, on the other hand, is related to the
342 formation or destruction of covalent bonds within the protein molecule, which modifies
343 the primary structure of the protein and therefore its structure and eventually its
344 bioactivity and therapeutic effect. The most frequent causes for chemical instability
345 include deamidation, oxidation, and cystine destruction or disulphide exchange. **Figure**
346 **4** represents the different physical and chemical sources of protein instability [37,39].
347 Therapeutic proteins are usually administered parenterally (intravenously,
348 subcutaneously, or intramuscularly) due to their high susceptibility to suffer proteolysis
349 in the gut and their difficulty to permeate membranes. Apart from drugs administered
350 intravenously, all drugs administered by other routes will have to permeate membranes
351 to be absorbed. The gastrointestinal tract (GIT) is the most important site for drug
352 absorption since oral administration is the preferred route of administration. GIT
353 permeation rate of compounds is dependent on the intestinal permeability and the
354 effective therapeutic protein available for permeation and its concentration in the GIT
355 fluid. Moreover, is further dependent on the specific physicochemical proprieties as
356 lipophilicity, molecular weight, size, and surface charge that influence the
357 pharmacokinetics and biodistribution of the protein.
358 There are several approaches to overcome the therapeutic protein delivery
359 challenges, which can be coupled into four categories: amino acid manipulation, post-
360 translation modification, bioconjugation, and carrier-mediated delivery.
361 The amino acid manipulation techniques consist of inserting, deleting, or altering
362 one or more amino acids in the protein chain, which has been proven to reduce
363 immunogenicity and proteolytic cleavage *in vivo*. Considering that the immunoglobulin

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364 G has a long-circulating serum half-life (~3 weeks) through pH-dependent FcRn binding-
365 mediated recycling, a study was performed to extend serum persistence of non-antibody
366 therapeutic proteins, by taking advantage of the intracellular trafficking and recycling
367 mechanism of IgG. The results showed an improvement in the serum half-life of
368 engineered FcγRIIa fusion, which suggests that this strategy has the potential to prolong
369 the half-life of therapeutic proteins [40]. One of the strategies employed to reduce renal
370 clearance rate is increasing protein size and molecular weight. Therefore, post-translation
371 modification consists of attaching the protein to polymers that can be either natural or
372 synthetic to increase their hydrodynamic volume, prevent rapid renal clearance and
373 thereby increase the protein serum half-life [41]. The proteins conjugates with more
374 clinical and commercial success have been with polyethylene glycol (PEG), a non-toxic
375 and non-immunogenic polymer approved for internal use (**Figure 5A**). PEG main
376 advantages are its solubility in both aqueous and organic solvents, presenting great
377 flexibility, high hydration that consequently increases its hydrodynamic volume, and a
378 range of molecular weight species allowing tuneable properties. All these proprieties are
379 also acquired by the therapeutic proteins bonded covalently to PEG, in a process called
380 PEGylation. The water cloud surrounding the protein conjugated with PEG may increase
381 solubility, become resistant to antibodies, proteolytic enzymes, and cells, and, due to their
382 increased size, are more slowly filtered by the kidneys [42].

383 The main foundation of bioconjugation approaches is that during hepatic
384 metabolism, proteins are taken by hepatocytes, receptor mediated. After that, they are
385 degraded in the lysosome by enzymes and cleared out of circulation. However, some
386 endogenous proteins can avoid liver metabolism, by imitating the specific receptor-
387 mediated recycling of endogenous proteins. Therefore, it was observed that binding of
388 therapeutic proteins to some endogenous proteins, as albumin or immunoglobulin,
389 receptor coupled recycling helps target protein recycle back to circulation as their
390 moieties and, therefore, it can be used as a strategy to avoid enzymatic degradation,
391 extending the half-life of therapeutic proteins (**Figure 5 B**) [41].

392 Carriers are used to protect the protein allowing its targeted and controlled
393 delivery [41]. The use of nanocarriers to deliver therapeutic proteins is deepened in the
394 following section**.**

395

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396 **4. Nanocarriers as tools to improve therapeutic proteins delivery**

397 The development of nanotechnology represents one of the most revolutionary and
398 promising technologies of the XX century. Nanoscience is the study of structures and
399 molecules on the nanometer scale, and nanotechnology is its practical application (Hulla
400 *et al.* 2015; Bayda *et al.* 2019). Nanotechnology is the manipulation and control of matter
401 on the nanoscale dimension, which ranges from 1 to 1000 nm, applied to several
402 industries and in biomedical scientific knowledge [45]. The prefix "nano" derives from
403 the Greek word that means "dwarf" or reduction in size, corresponding to a one thousand
404 million of a meter reduction. This reduction, along with the ability to control and
405 manipulate structures in nanoscale enables the exploration of new physical, biological,
406 and chemical properties of systems [46].
407 In 1959 the physicist and Nobel Prize winner Richard Feynman first introduced
408 the concept of nanotechnology when he presented a lecture entitled "There's Plenty of
409 Room at the Bottom" at the California Institute of Technology, proposing the hypothesis
410 "Why can't we write the entire 24 volumes of the Encyclopaedia Britannica on the head
411 of a pin?" to explain his vision of using machines to construct smaller machines, down to
412 the molecular level [47]. Ever since, a great advance has been made and nanotechnology
413 is now applied in several areas as physics, chemistry, computer science, and biology.
414 Several studies proved the enormous potential of nanotechnologies in biomedicine for the
415 diagnosis and treatment of several diseases, with significant advances in this field,
416 especially for cancer treatment due to the potential to overcome the limitations of the
417 traditional approaches [48].
418 Drug discovery is a time-consuming, arduous, expensive, and high-risk process,
419 with a significantly low success rate and several challenges to overcome. Furthermore, in
420 the last decades, it became evident that drug development alone is not enough to secure
421 progress in drug therapy. The main reasons for therapy failure include insufficient drug
422 concentration due to pharmacokinetics proprieties, and inconstant plasma levels because
423 of the pharmacodynamics influence. It is also due to the lack of specificity of some drugs
424 and poor drug solubility. Recognizing these aspects, the development of suitable drug
425 carrier systems emerged as a promising solution [49,50].
426 Drug delivery systems are used to enable controllable drug release and improve
427 both its safety and efficacy. Nanotechnology has begun to be implemented for this
428 purpose and other than satisfying the mentioned goal of drug delivery systems, also

429 targets the loaded drugs into specific body locations. Consequently, the main objectives
430 of nanotechnologies include more specific drug targeting and delivery, reducing toxicity
431 while maintaining therapeutic effects, enhancing safety and biocompatibility, and
432 accelerating the new medicines development process. Even though drug delivery systems
433 do not modify the pharmacokinetic or pharmacodynamics of the drug, they can modulate
434 it, enabling long-acting therapeutic formulations. The mentioned modulation is based on
435 the concept of incorporating the protein into a matrix or into another molecule that will
436 work like a protective covering. This cover will also function as a depot that instead of
437 releasing all the therapeutic at once will gradually release it in circulation, creating a long-
438 acting formulation [17,51].

439 Nanoparticles are attractive as drug delivery systems due to their unique
440 characteristics as the surface to mass ratio is higher when compared with other particles,
441 ability to adsorb and carry other compounds such as drugs and proteins, and enhanced
442 solubility and diffusivity. All these characteristics of nanoscale materials and the
443 enhanced solubility and diffusivity have been proven to increase the blood circulation
444 half-life [42]. As mentioned, the size of the nanoparticles ranges from 1 to 1000 nm, but
445 for nanomedicine purposes, sizes smaller than 200 nm are preferable due to the ability to
446 traverse micro-capillaries. Still, particle sizes above 100 nm may be required for loading
447 enough drugs [51,52]. Apart from the advantages, there are also significant disadvantages
448 to the use of nanoparticles. Burst release of the therapeutic and the consequent side
449 effects, poor loading efficiency, and manufacturing and administration challenges are
450 some of the most frequent [17,53].

451 There are several classifications of nanoparticles according to their morphology,
452 size, and chemical properties (**Figure 6**). Considering the composition materials of the
453 nanoparticles they can be divided into categories:

454 (1) Carbon-based nanoparticles – Fullerenes and carbon nanotubes (CNTs) are the
455 biggest subclasses. Fullerenes are composed of a globular hollow cage form of pentagonal
456 and hexagonal carbon units, especially interesting due to their electrical conductivity,
457 high strength, structure, electron affinity, and versatility. CNTs are elongated tubular
458 structures, structurally like a rolling graphite sheet. These are frequently used for
459 commercial applications fillers and efficient gas adsorbents for environmental purposes
460 [54–56].

461 (2) Metallic nanoparticles – Made by metals precursors with unique optoelectrical
462 properties which make them valuable for applications in research areas [55].

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463 (3) Polymeric nanoparticles – Usually organic-based nanoparticles, in their majority
464 with nanosphere or nanocapsules shape with a wide range of applications [57,58].
465 (4) Lipid-based nanoparticles – Made of lipids both in a solid or liquid state. It is fully
466 addressed in the next section.
467 The latter are the focus of this review and are fully disclosed in the next section.
468

469 **5. Lipid nanoparticles for therapeutic proteins delivery**

470 The use of lipid nanoparticles as drug carriers have been studied for years. The first
471 emulsion introduced as carrier systems, in the fifties, was only intended to reduce the
472 drug side effects. Although accomplishing the intended goal, they did not have the
473 expected success, which can be explained by the physical instability caused by the
474 incorporated drug and the low solubility of the used lipids. Later, in 1965, liposomes were
475 developed by Bangham and introduced as drug delivery systems in 1986 by Dior® in the
476 cosmetic market. Few years later, at the end of the eighties, liposomes started being used
477 in the pharmaceutical field as drug delivery systems. Even so, and same as for the O/A
478 emulsions, the number of products on the market is still limited, in part due to the
479 excessive cost of pharmaceutical liposomes [59].
480 Other lipid based systems for encapsulation of therapeutic drugs have been
481 extensively used specifically for topical drug administration once their lipid bilayers
482 mimic the human cell membrane, to the delivery of cosmetics like vegetable oils and
483 therapeutic factors that promote wound healing [60-62]. They are used also for oral
484 delivery to encapsulate unstable compounds like antimicrobials, antioxidants, flavors,
485 and bioactive elements to preserve their functionality [61,63]. Finally, the advantage of
486 ocular therapy by topical administration is not less important than the others. Lipid based
487 systems can increase the internalization of the drugs, higher permeation, increase
488 precorneal residence time, and sustained drug release with a minimum dosing frequency
489 and decreased drug toxicity, which consequently promotes a higher improvement in
490 ocular drug bioavailability and therapeutic success. Also, its nanometric size reduces the
491 clearance by the eye's protective mechanisms due to its adhesive properties [64–66].
492 On another hand, the effectiveness of therapeutic proteins depends on their
493 bioavailability, which can be defined by the ability of a compound to reach the site of
494 action at a rate and amount necessary to illicit the therapeutic effect. For most drugs, the
495 therapeutic effect is related to the plasma levels which means that the term bioavailability

496 can be defined as the rate and extent of absorption of unchanged drugs from their dosage
497 form [56,57]. The use of lipid nanoparticles has several advantages including
498 improvement of bioavailability and others [67,68]:

499 - Biocompatibility and biodegradability
500 - Low toxicity
501 - Targeted and controlled drug release
502 - Encapsulation of both hydrophilic and hydrophobic compounds
503 - Ease scalability of production methods

504

505 Therapeutic proteins are highly vulnerable molecules due to their physical and
506 chemical instability. They are often administered intravenously to overcome their short-
507 half life. Furthermore, they also present a poor capacity of penetrating membranes, which
508 is a considerable limitation for their administration by other administration routes. The
509 use of lipid nanoparticles as drug delivery systems allows overcoming these limitations
510 of therapeutic proteins. The lipid nanoparticles structure protects the therapeutic protein
511 structure from degradation, but it also increases their bioavailability and capacity to
512 penetrate membranes. The lipid nanoparticles allow the entrapment of both lipophilic and
513 hydrophilic compounds like proteins and fulfil the requirements to be used as an optimal
514 drug delivery system. The encapsulation of therapeutic proteins into lipid nanoparticles
515 can address the major limitations of the therapeutic proteins and open a completely new
516 window of opportunities [8].

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

519

520 ### *5.1. Liposomes*

521 Liposomes, an early version of lipid-based nanoparticles, are composed by lipids
522 and fatty acids that are considered biocompatible and biodegradable owing to their natural
523 occurrence in cell membranes. Additionally, their structure has early attracted the
524 attention as a promising delivery system due to its flexibility, low immunogenicity, low
525 toxicity, easy preparation, extended circulation time, and the ability to extend the shelf
526 life of formulations [61,69].

527 Liposomes are small vesicles of spherical shape with particle sizes ranging from
528 100 nm to 1000 nm composed by at least two lipophilic layers [70]. These nanostructures

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529 are defined by the spontaneous assembly of phospholipids into a bilayer sphere, in which
530 the hydrophilic head groups face the exterior aqueous environment, and the hydrocarbon
531 chains assemble within the hydrophobic interior (**Figure 7**). It was the first lipid
532 nanostructure to be produced due to its self-production capacity owing to its amphiphilic
533 character, since in aqueous solutions phospholipids impulsively form closed structures
534 [70]. Their amphiphilic character also allows it to be a versatile drug delivery system for
535 both hydrophobic and hydrophilic drugs [61,69]. Hydrophobic drugs are carried between
536 the phospholipid layer, and hydrophilic in the aqueous core of the liposome [71].

537 Liposomal encapsulation of therapeutic drugs has been extensively used. In this
538 sense, liposomes may be applied for protein delivery while keeping their structure and
539 bioactivity. Water soluble proteins can be carried inside the aqueous core or can be
540 attached to the lipid surface, while hydrophobic peptides or proteins are inserted into the
541 inner hydrophobic center of the bilayer [72].

542 Dawoud *et al.* loaded insulin into a chitosan-based spray in liposomes intended
543 for wound healing by topical delivery. Different lipids were used and the effects of the
544 cholesterol addition, method of preparation, and sonication were evaluated on the particle
545 size and the entrapment efficiency. Liposomal insulin particle sizes ranged from 0.7 to
546 2.9 μm, depending on the use of cholesterol, since this lipid increased the diameter of the
547 vesicle. The encapsulation efficiency of insulin varied between 37% and 84% depending
548 on the preparation technique and the presence of sonication, which decreased the amount
549 of the loaded drug. Finally, studying the behavior in Franz diffusion cells, the insulin
550 dispersion, and the optimized liposomes formulation revealed a prolonged release of 6 h
551 and up to 24 h, respectively. These findings revealed that topical insulin liposomal spray
552 offered a protective method for insulin delivery [73].

553 Another formulated liposomal drug is the patented vaccine Epaxal®, a liposomal
554 nanoparticle formulation of a protein antigen used as a hepatitis A vaccine, in which the
555 viral envelope glycoproteins are intercalated in the phospholipid bilayer membrane. This
556 structure facilitated the delivery of hepatitis A virus antigen to immunocompetent cells
557 given the properties of the active fusion glycoproteins [74].

558 However, in some cases, protein delivery by liposomes has a rapid clearance by
559 the mononuclear phagocyte system. One strategy to overcome this problem is the
560 conjugation of the lipid surface with an inert polymer such as PEG. The steric impediment
561 effect of PEG chains resulted in the increase of the hydrodynamic volume of the system,
562 and the PEG capacity to avoid the immune response. However, it is important to avoid a

563 high degree of conjugation with PEG as this results in a reduction in the melting
564 temperature of the liposomes, which promotes their destabilization. So, PEGylation may
565 just extend their circulation half-life from 30 min to 5 h [62,72].

566 Based on their size and number of bilayers, liposomes can be classified into three
567 categories [61,71,75]:

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

573 Usually, different unilamellar vesicles encircle inside each other with successively
574 smaller sizes, creating a multilamellar structure of concentric phospholipid spheres
575 separated by layers of water [75].

576 The production method defines the type and size of liposomes produced and those
577 influence drug encapsulation efficiency and circulation time [71]. Different production
578 methods, as well as lipid compositions, can be used influencing the properties of these
579 nanosystems, namely surface charge and size. The surface charge of these nanostructures
580 is usually determined by the charges of the lipid head groups, which can be positively or
581 negatively charged or zwitterionic. This surface charge influences the interactions
582 between particles and the adsorption of counterions, and thus the stability of the
583 nanoparticles. Thus, uncharged particles or particles with low charge tend to aggregate
584 over time, while more highly charged particles repel each other, preventing aggregation
585 [76].

586 In addition, the type of bilayer components used in their preparation influence the
587 'stiffness' or 'fluidity' and the charge of the bilayer. An example of this is unsaturated
588 phosphatidylcholine species from natural sources (phosphatidylcholine from eggs or soy)
589 result in bilayers that are much more permeable and less stable, whereas saturated
590 phospholipids with long acyl chains (e.g., dipalmitoylphosphatidylcholine) form a rigid
591 and impermeable bilayer structure [61]. Other types of lipids are used in liposome
592 preparations which allow modulating the nanostructure properties (**Table 3**).

593 However, liposomes revealed some drawbacks concerning stability problems over
594 time such as easy sedimentation, aggregation, and coalescence that can shorten their
595 shelf-life, resulting in loss of liposome-associated drugs, and changes in size. These issues
596 lead to low reproducibility, reduced encapsulation efficiency, high polydispersity index,

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597 and unexpected and uncontrolled drug release during storage. In another hand, sometimes
598 phospholipids undergo oxidation and hydrolysis-like reaction. In this sense, is crucial to
599 control their stability during and after the production process [69,77]. Moreover,
600 liposomes can suffer accumulation in liver and splenic macrophages, leading to
601 splenomegaly and hepatotoxicity [78]. Therefore, due to the natural instability of
602 liposomes, which limits their clinical use among other disadvantages, these
603 nanostructures showed not to be robust enough for the delivery of proteins, so other lipid
604 based nanocarriers were developed. **Table 4** summarizes some more recent works.
605
606 ***5.2. Solid lipid nanoparticles***

607 SLN were developed by Schwarz *et al.* (1994) and in parallel by Morel *et al.*
608 (1998) [79,80]. SLN brought attention due to its advantages, being able to assemble the
609 advantages of other colloidal carriers while avoiding some of their disadvantages. These
610 nanoparticles are interesting delivery systems that have shown great advantages
611 including:
612 • Allowing controlled drug release and targeting
613 • Increasing drug stability
614 • Allowing high drug payload
615 • Incorporating lipophilic and hydrophilic drugs
616 • Being composed of biocompatible lipids
617 • Large-scale production ability
618 • Use of Generally Recognized as Safe (GRAS) compounds and therefore low
619 toxicity as carriers
620 • Improvement of drug stability and safety
621
622 They have been actively investigated for the delivery of drugs by different
623 delivery routes [50,81,82]. The most frequently used excipients for SLN production are
624 shown in **Table 5.**
625 The lipids compose the matrix of the SLN, and are solid at room and body
626 temperature, usually with a melting point above 40ºC, used in a concentration ranging
627 from 5 to 40%. Distinct types of lipids are used, ranging from triglycerides, partial
628 glycerides, and fatty acids to steroids and waxes. This fact is one of the major advantages
629 of SLNs, as they are made of physiologic materials decreasing the danger of acute and

630 chronic toxicity of these nanoparticles. The choice of lipids relies on the solubility of the
631 compound that is incorporated inside the matrix [63,83].
632 The emulsifier role in the formulation is to reduce the surface tension between the
633 aqueous and lipid phases, thereby helping the stabilization of the system. Since they are
634 amphiphilic molecules, they are placed in the interface of the system [50,81]. Several
635 types of emulsifiers have been employed in SLN formulations, like as bile salts,
636 ethoxylated alcohols, fatty acids, phospholipids, poloxamers, polyethylene glycols,
637 polysorbates, polyvinyl alcohols, quaternary ammonium compounds, sorbitan esters, and
638 tyloxapol, and it was discovered that a binary combination of emulsifiers helps to stabilize
639 the systems more effectively and results in smaller nanoparticle sizes [63]. The choice of
640 emulsifiers should take into consideration the hydrophilic-lipophilic balance (HLB) of
641 the lipids employed in the formulation, as well as their concentration of the lipid phase
642 and the administration route [50,81].
643 There are three incorporation models for the SLN that differ in the location and
644 distribution of the loaded therapeutic protein within the lipid core as shown in **Figure 8.**

645 - **SLN Type I/homogeneous matrix model –** In this model the drug is dispersed
646 in the lipid core or as amorphous agglomerates. This type is usually produced by
647 high pressure homogenization (HPH), either cold HPH or hot HPH with an
648 optimized drug/lipid ratio. Usually, these nanoparticles show good controlled
649 release properties.
650 - **SLN Type II/drug enriched shell model** - In this model it is obtained a drug-
651 free lipid core surrounded by an outer shell containing the drug and the lipid. This
652 model is used when a faster release of the encapsulated drug is desired.
653 - **SLN Type III/drug enriched core model** – In this model, the core of the
654 nanoparticle is enriched with drug while the lipid is in the outer shell. This
655 morphology is obtained when the drug concentration in the melted lipid mass is
656 close to its saturation solubility and the lipid, when cooled, precipitates in the core
657 before the lipid. This model is also suitable for drugs that require a prolonged drug
658 release [68,84].

659 Nevertheless, the SLN has two main limitations related to its densely packed
660 crystal structure: low loading capacity and drug expulsion during storage. Both lipophilic
661 and hydrophilic active substances can be entrapped [63]. Drugs are mostly incorporated
662 between the fatty acid chains, lipid layers, or in the amorphous clusters of the crystal
663 imperfections. SLN usually crystallizes in a perfect lattice, especially those obtained by

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664 highly purified lipid, which explains the low encapsulation efficiency, since the more
665 densely packed the crystal is the less drug is possible to incorporate [85]. Furthermore,
666 during storage, the lipid molecules suffer a time-dependent restructuration process in
667 which the more perfect lipid crystalline structures lead to the expulsion of the drug [86].
668 Additionally, SLN dispersion may suffer gelation phenomenon once its viscosity
669 increases during the cooling process which results in a viscous gel and consequently leads
670 to an increase in particle size and particle agglomeration [63].
671 In a study where bovine serum albumin (BSA) was used as a model protein for
672 the encapsulation into a matrix modified by incorporation of lecithin into the lipid matrix
673 and different emulsifier concentrations, the obtained particle payload with BSA was
674 between 2.5 and 15% and seemed to be commanded by the particle surface
675 characteristics, particularly the surface charge and the specific surface area [87]. In a
676 different study, using SLN for the encapsulation of lysozyme, the method used produced
677 formulations with reduced concentration of protein and low encapsulation efficiency,
678 which considering the excessive costs of some therapeutic proteins and the waste
679 generated by a reduced encapsulation efficiency, represents a limiting factor, and urges
680 the need of developing of further improved lipid nanoparticle formulations [88]. In **Table**
681 **4** it is summarized more works focusing on SLN for protein delivery.
682
683 ### *5.3. Nanostructured lipid carriers*

684 NLC were developed to overcome the main limitations of the SLN that could
685 compromise the applicability of the formulation: the low drug loading capacity and drug
686 expulsion during storage. Therefore, it was investigated possibilities to improve the SLN
687 formulation, being discovered that adding a liquid lipid into the solid matrix of the SLN
688 increases the imperfections on the matrix, which leads to a higher loading capacity while
689 maintaining the stability of the formulation. The structural differences between SLN and
690 NLS are shown in **Figure 9**. Therefore, the NLC are composed of an unstructured solid
691 matrix composed of a mixture of solid and liquid lipid, and an aqueous phase containing
692 one or more surfactants. In general, the lipids are mixed in a 70:30 up to 99.9:0.1
693 solid/liquid ratio and the concentration of the surfactant ranges from 1.5% to 5% (w/v).
694 The excipients employed in the production of NLC are the ones used for SLN plus a
695 liquid lipid [68,81,89] – **Table 5.** The liquid lipid could be fatty alcohols, medium-chain

696 triglycerides, paraffin oil, and squalene. Moreover, fatty acids, such as oleic, linoleic, and
697 decanoic acid may be used since their properties as penetration enhancers [83].
698 Same as the SLN, according to the production method, and the composition of the
699 lipid mixture, there are three types of NLC (**Figure 10**).

700 - **The imperfect type** – Occur when spatially different lipids are mixed, composed
701 of fatty acids that introduce imperfections in the crystal matrix. These
702 imperfections allow a higher drug loading capacity, which can be further
703 increased by using different glycerides and varying the saturation and length of
704 the carbon chain.
705 - **The amorphous type** – In this type, it is used solid special lipids as
706 hydroxyoctacosanyl hydroxy stearate or isopropyl myristate with a liquid lipid,
707 forming a structureless amorphous matrix. The resulting amorphous state instead
708 of an ordered state avoids β-modification during storage and therefore the drug
709 expulsion.
710 - **Multiple oil-in-solid fat-in-water (O/F/W) type** – This last type results in
711 numerous nanosized liquid oil compartments disseminated in the solid matrix. In
712 this case, the drug solubility is higher in the oil compartments, which increases
713 the loading capacity and the prolonged release because the compartments are
714 surrounded by solid lipids [68,84].

715 In the last years, the number of papers on NLC formulations increased
716 considerably and it has been emerging as an ideal drug delivery system for the
717 pharmaceutical market. However, studies addressing the delivery of therapeutic proteins
718 by NLC are scarce, due to formulation production challenges.
719 In a recent study, it was developed coenzyme Q10-loaded (co-Q10) NLC by the
720 high shear homogenization method, obtaining spherical nanoparticles with an average
721 particle size of 180-350 nm, a PdI below 0.5, zeta potential below -0.3 mV and an
722 encapsulation efficiency between 83 to 88% [90]. Nevertheless, there is no description in
723 the literature of therapeutic proteins encapsulated into NLC or produced by methods that
724 would not damage the protein tridimensional structure.
725 Another study developed by Rocha *et al.* proposed the use of nanostructured
726 systems to enhance the antimicrobial activity of antibiotics, namely the polypeptide
727 bactericidal antibiotic Polymyxin B by functionalization. NLC loading dexamethasone
728 acetate and its surface were modified by polymyxin B sulfate were developed intended
729 to increase the antimicrobial activity against *P. aeruginosa*. NLC was obtained by high-

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730 pressure homogenization and coated with polymyxin B. The produced formulation
731 revealed good stability and physicochemical characteristics and this new carrier platform
732 showed an enhanced polymixin B antimicrobial activity 2- to 3-fold against *P. aeruginosa*
733 revealing that this peptide conjugation strategy may be a new successful treatment against
734 gram-negative bacterial infections [63].

735 In the literature, there is not much more description of NLC for the encapsulation
736 of therapeutic proteins **(Table 4).** This is mainly because production methods usually use
737 temperature and pressure, which is not suited for the encapsulation of therapeutic
738 proteins. From the several methods available to produce NLC, the HPH and
739 microemulsion techniques are the preferred methods [85,91]. Since NLC are obtained by
740 emulsification, it is necessary to have both the lipid and the aqueous phases in the same
741 physic state, which can be obtained either by melting the lipid or dissolving it in an
742 organic solvent. Avoidance of organic solvents is preferable, but for therapeutic proteins,
743 the employment of temperature is not the best option because it can damage the protein
744 structure.

745 With the emerging importance of therapeutic proteins, and all the advantages
746 previously mentioned, their encapsulation into NLC using a method that do not damage
747 the protein structure, can change the paradigm of therapeutic proteins, allowing their
748 administration by different delivery routes and optimize their use as therapeutics.
749

750 **6. Production methods of therapeutic proteins-loaded lipid nanoparticles**

751 ***6.1. Liposomes production***
752
753 Liposome production methods, in general, involve the following steps: the
754 extraction of lipids from the organic solvent; their dispersion in an aqueous solvent or
755 buffer; the purification of the liposomes formed; and the analysis of the final product
756 [61,92]. During preparation, the types and amounts of phospholipids, the ionic and
757 polarity properties of the aqueous medium, and the techniques used are crucial factors
758 that determine the final structure of the liposome. The encapsulation of therapeutic
759 proteins occurs passively during liposome formation or actively after liposome
760 preparation. Among the main passive loading techniques, the mechanical dispersion
761 methods (sonication, extrusion, freeze-thaw, thin film hydration, and microfluidization),
762 solvent dispersion method and removal of detergent or non-encapsulated material are the

763 main ones. The isolation of liposomal vesicles is based on the principles of dialysis,
764 adsorption, gel permeation chromatography, and dilution [61,93].
765

766 **6.1.1. Sonication**

767

768 Sonication is the most frequently used technique to prepare small ULVs. For the
769 preparation of MLVs sonication occurs in a bath-type sonicator, or a probe sonicator
770 under a passive atmosphere. The protein solution is added to the surfactant and cholesterol
771 solution, while the mixture is sonicated at the surfactant transition temperature for
772 minutes. However, this method presents low encapsulation efficiency, and may enhance
773 the degradation of phospholipids and drugs to be encapsulated [61,94].
774

775 **6.1.2. Extrusion**

776

777 The extrusion method involves passing the suspension of MUVs through a
778 membrane filter of defined pore size to form small ULVs, being more suitable for labile
779 materials, like proteins, than sonication [95]. The application of high pressure helps the
780 extrusion of the vesicles through the small polycarbonate pores transforming large
781 vesicles into small ones due to the passage through the pores. The properties of the
782 liposomes formed namely, the average size and polydispersity, depend on the applied
783 pressure, the number of cycles, and the pore size of the filters used. This is a simple, fast,
784 and reproducible method that gives rise to homogeneous size distributions. Thus, the
785 main disadvantages of this method are blockage of the pores, possible loss of product,
786 and the working volumes are relatively small [94,96,97].
787

788 **6.1.3. Thin film hydration**

789

790 In this method, the surfactants and lipid molecules are solubilized in an organic
791 solvent or a mixture of volatile organic solvents. Then, by reducing the pressure the
792 solvent is evaporated, leaving a thin film of lipids. Then, a large volume of protein
793 aqueous solution is added slowly to the film on the inner surface of the container at a
794 temperature above the transition temperature of the lipid used. The volume of the aqueous
795 solution used and this hydration step, where one phase interacts more with water than the
796 other, influences the properties of the liposomes. High water volumes lead to the

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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 [69,94].

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 minutes together with the therapeutic protein. This is followed by a rapid transfer to a water bath at surfactant transition temperature for 5 minutes. 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 [61,69,94].

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 [94,97].

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 [95].

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 **Figure 11** and further detailed in this section [81,98]. 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 [87].

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 100-2000 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 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 (**Figure 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 5ºC above the lipid melting point [50,81]. The hot HPH is less adequate for the encapsulation of therapeutic proteins due to the elevated temperatures applied during the emulsification

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865 process. The cold HPH can be considered as an option for the encapsulation of therapeutic
866 proteins if the pressure applied is managed to not damage the therapeutic protein.
867 Lysozyme was used as a model protein for optimizing the incorporation of
868 therapeutic proteins into SLN using both variations of the HPH method. The results
869 showed that protein remained intact during all the harsh conditions of the procedure, but
870 the encapsulation efficiency was only about 59%, because the protein tended to partition
871 to the aqueous phase. These results were not completely surprising because the lysosome
872 is a protein with high structural stability [8,88]. On the other hand, results using BSA
873 showed the temperature and pressure conditions of HPH strongly influenced the protein
874 structure. Other studies using human insulin and cyclosporine A showed both proteins
875 maintained their structures, with cyclosporine A showing an incorporation efficiency
876 above 90% [8].
877

878 **6.2.1.1 Hot high pressure homogenization**

879 In this technique, the entire process is performed at temperatures above the lipid
880 melting point, and under high shear stirring a pre-emulsion is prepared. The lipid melt,
881 and the aqueous emulsifier are mixed, both at the same temperature. After that, the formed
882 pre-emulsion is homogenized by HPH. Hot temperatures frequently lead to lower particle
883 size but, on the other hand, they can also increase the degradation rate of the system.
884 Usually, one cycle of homogenization is enough to produce SLN and NLC with an
885 average particle size ranging from 250 to 300 nm, when the pre-emulsion concentration
886 is between 5-10%. When the concentration is higher than 30% is no longer possible to
887 produce NLC, but highly concentrated SLN can still be obtained. In these cases, it is
888 important to adjust the number of homogenization cycles since the energy required to
889 shear the lipid mass is proportional to its concentration in the formulation. However, it is
890 important to notice that increasing the number of homogenization cycles also frequently
891 leads to bigger particle size, because increased particle kinetic energy, favors
892 coalescence. At this stage, an emulsion is obtained due to the physic state of the lipid.
893 The last step is cooling the sample at room temperature or lower, leading to lipid
894 crystallization and formation of the nanoparticles [50,81].
895 According to the literature, this technique can be used for some heat-sensitive-
896 compounds because the time of exposure to elevated temperatures is short. Even so, the
897 temperature employed is one of the limitations of this technique since, as mentioned, it is

898 unsuitable for overly sensitive compounds and hydrophilic compounds that in elevated
899 temperatures can partition from the lipid phase to the aqueous phase [50,81].
900 Nevertheless, there is no relevant works in the literature of therapeutic proteins
901 encapsulated into lipid nanoparticles using this production method. The reason for this is
902 because therapeutic proteins are highly temperature sensitive compounds, and therefore
903 the use of temperature would damage the structure of the proteins and compromise its
904 therapeutic effect.
905

906 **6.2.1.2. Cold high pressure homogenization**
907

908 This technique was developed to overcome the limitation of hot HPH: degradation
909 of the loaded bioactive due to elevated temperature exposure, drug partition into the
910 aqueous phase and the complex crystallization step being recommended for extremely
911 heat sensitive and hydrophilic compounds, by reducing the temperature exposure. After
912 the preparatory step, the obtained mixture is rapidly cooled down to a solid state, using
913 dry ice or liquid nitrogen, favoring a homogenous distribution of the drug. Then, the
914 obtained solid is turned into microparticles. First, a pre-suspension is prepared by
915 dispersing the obtained microparticles in a cold emulsifier solution and then, the mixture
916 is subjected to HPH, at or below room temperature, forming the lipid nanoparticles. For
917 this method, five cycles at 500 bars are usually performed to obtain SLN and NLC.
918 The main disadvantage of this technique is the need to employ high energy during
919 the homogenization step. Also, the particles formed are usually bigger and more
920 polydisperse than those formed using the hot HPH [50,81].
921

922 **6.2.3. Emulsification methods**
923

924 The emulsification methods are the best to load therapeutic proteins into lipid
925 nanoparticles, due to the avoidance of elevated temperature and shearing stress. In this
926 section different emulsification methods are addressed.
927

928 ***6.2.3.1. Ultrasonication***
929

930 Ultrasonication is a dispersing technique, on which the lipid nanoparticles are
931 obtained by dispersing the melted lipid phase in the aqueous phase with the surfactant. It
932 allows the cleavage of large particles into smaller ones, by providing energy, usually
933 above 20 kHz of ultrasonic rates/frequencies for homogenization [68,99]. In the first step

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934 of this technique, the lipid is melted, about 5-10ºC above its melting point. Then, the melt
935 is dispersed in an aqueous surfactant, at the same temperature, under high stirring to form
936 an O/A emulsion. The formed emulsion is subjected to sonication to reduce the droplet
937 size. In the last step, the emulsion is cooled at a temperature under the solidification
938 temperature of the lipid, with the formation of a nanoparticle dispersion [68]. Some of
939 the advantages of this technique relate to the equipment used, which are common
940 laboratories material. However, the energy distribution during sonication is not
941 homogenous, resulting in highly polydisperse particles [81].

942 In a previous work, to evaluate the influence of sonication time and pulse
943 frequency on average dispersion, temperature, particle size and zeta potential, SLN were
944 prepared using a 1:3 ratio of stearyl alcohol (SA) and cetyltrimethylammonium bromide
945 as lipids, applying different sonication times and pulse frequencies, respectively 5, 10 and
946 15 min and 30, 60 and 90%. The values were selected based on the results from a
947 preliminary study. During the sonication process, only the pulse frequency and sonication
948 time were varied, maintaining all the other parameters constant. The desired SLN size
949 was about 100 nm, which was obtained with 60% pulse frequency at 40% power for 10
950 min. These optimized sonication parameters were used to study the influence of the lipid
951 on size and zeta potential, applying the same parameters using different lipids. The
952 resulting SLN were after evaluated to determine the short-term stability in aqueous
953 dispersions. The mean particle sizes of SLNs made of SA, cetyl palmitate, Precirol,
954 Dynasan118 and Compritol were about 98, 190, 350, 350 and 280 nm, respectively. The
955 obtained results suggested that an increase in pulse frequency and sonication time
956 produces smaller nanoparticles, unwanted increase in dispersion temperature but an
957 irrelevant influence on zeta potential. It was also found that increasing the length of the
958 hydrocarbon tail of the lipids increases the size of the nanoparticles [100].

959

960 ***6.2.3.2. Double emulsification***

961 This approach consists of emulsifying a heated aqueous solution of the drug in the
962 previously melted lipid, forming water in oil (w/o) emulsion, stabilized with proper
963 excipients. Then, the formed w/o emulsion is dispersed in an aqueous solution of a
964 hydrophilic emulsifier, forming a double water-in-oil-in-water (w/o/a) emulsion. Finally,
965 the emulsion is cooled under stirring, forming the solid lipid nanoparticles. This technique
966 is suitable for the incorporation of hydrophilic therapeutic proteins but, on the other hand,

967 the obtained particles are large [68]. In a study using cetyl palmitate, glyceryl tripalmitate
968 and glyceryl palmitostearate as the lipids for the preparation of SLN, using the double
969 emulsion technique, the nanoparticles were successfully prepared and their size were
970 447.5 ± 50.8, 444.8 ± 72.5, and 213.7 ± 38.4 nm, respectively [101].

971 In another study using thymopentin and insulin as the model protein drugs, it was
972 prepared a novel Gel-Core-solid SLN using a double emulsion technique. The goal of this
973 work was to enhance the entrapment efficiency, and it was favourably obtained the Gel-
974 Core-SLN with a particle size of 305.2 nm and zeta potential of -17.15 mV. The
975 entrapment efficiency of thymopentin-loaded Gel-Core-SLN and insulin-loaded Gel-
976 Core-SLN were 61.97% and 57.36%, respectively, with both presenting low burst release.
977 In terms of pharmacological availability of insulin-loaded Gel-Core-SLN the value was
978 6.02%. Therefore, this study showed promising results for the Gel-Core-SLN as a drug
979 delivery system prepared by a double emulsion technique [102].

980

981 ***6.2.3.3. Microemulsification***

982 The first step of this homogenization technique consists of placing both phases at
983 the same temperature, by melting the lipid or blend of lipids and heating the aqueous
984 phase containing the surfactant. Once both phases are at the same temperature, the
985 aqueous solution is added to the lipid solution, under mild stirring, to create the
986 microemulsion. Then, to obtain the microemulsion the system is dispersed in chilly water
987 with a temperature ranging from 2 to 10ºC, under mild mechanical mixing, thus ensuring
988 that the reduced particle size is due to the precipitation and not because of the mechanical
989 stirring process. The last steps are washing the system using distilled water, filtering it to
990 remove the larger particles and finally lyophilizing the system to remove the excess water
991 [103]. The big advantage of this technique is allowing the preparation of the particles
992 under mild temperature and pressure conditions. Some of its disadvantages are the need
993 for a high concentration of surfactant, the dilution of the system and therefore obtention
994 of a relatively dilute system, with low particle concentration [7,81].

995 The first attempt to encapsulate peptide drugs in SLN was carried by Morel et al.
996 (1994) using this technique for the encapsulation of triptorelin and thymopentin as model
997 peptides [104,105]. The encapsulation efficiency was low in both cases and equivalent
998 results were observed for the encapsulation of cyclosporine A [8].

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1000 **6.2.4. Solvent evaporation**
1001
1002 In this method, nanoparticle dispersion is obtained by precipitation of o/w
1003 emulsions. First, the lipophilic compounds are dissolved in an organic water-immiscible
1004 solvent. The obtained mixture is then emulsified in an aqueous phase, forming an o/w
1005 emulsion. The organic solvent is then evaporated, under reduced pressure, leading to the
1006 precipitation of the lipid in the aqueous medium and subsequent formation of a
1007 nanoparticle dispersion [50,106]. This approach also avoids temperature and high-energy
1008 sources, and it results in particles with a narrow size distribution [81,107].
1009 Overall, this is a widespread method in the preparation of nanoparticles, including
1010 SLN. For hydrophilic compounds, including proteins, associating the double emulsion
1011 technique to this method was demonstrated to improve their encapsulation efficiency.
1012 Thus, a big part of the studies with protein encapsulation in solid lipid nanoparticles is
1013 based on this method because it also avoids the use of temperature or pressure conditions.
1014 However, the use of organic solvents can increase the toxicity of the final product.
1015 This method was used for the encapsulation of insulin, resulting in a 45% burst
1016 release. The same authors using calcitonin as model were able to demonstrate the
1017 feasibility of the method, obtaining encapsulation efficiencies above 90%. In a study
1018 conducted to improve the oral absorption of insulin, an insulin-loaded Vitamin B12
1019 (VB12)- gel core solid lipid nanoparticles (GCSLN) were prepared by a combination of
1020 double emulsion and solvent-evaporation methods. The results of this study were very
1021 promising for the use of VB12-GCSLN containing insulin as a carrier for drug delivery.
1022 The VB12-GCSLN had an encapsulation efficiency (EE) of 55.9%, a burst release of less
1023 than 10% in the first 2 h, an absorption of insulin with a relative pharmacological
1024 availability of 9.31% and considerable stable blood glucose levels up to 12 h [108].
1025
1026 **6.2.5. Solvent injection**
1027
1028 In this method a transitional o/w emulsion is prepared using a partially water-
1029 soluble solvent that is firstly saturated in water, to guarantee initial thermodynamic
1030 equilibrium. The fundament of the technique is the partial solubility of the compounds in
1031 water. First, the lipids are dissolved in a water-miscible solvent forming a mixture rapidly
1032 injected by an injection needle, into an aqueous surfactant solution under continuous
1033 stirring, causing the organic solvent to diffuse into the water, leading to droplet size
1034 decrease and consequent formation of the nanoparticles [68]. This method uses mild

1035 organic solvents, avoids several critical as elevated temperatures, high pressures, and high
1036 emulsifier concentrations, and has emerged as an efficient, versatile, and easy to
1037 implement technique [81,109].

1038 The solvent injection method was firstly used to produce lipid nanoparticles by
1039 Schubert *et al.* (2003). The results from this study showed that acetone, ethanol,
1040 isopropanol, and methanol are suitable solvents for the preparation of lipid nanoparticles,
1041 which was not verified with ethyl acetate that was not able to successfully produce the
1042 nanoparticles. The particle sizes obtained were 80-300 nm depending on the preparation
1043 conditions. It was also performed a physicochemical characterization of the particles that
1044 revealed a decrease in crystallinity of the colloidal lipid when compared to the bulk lipid
1045 [109].

1046

1047 **7. Conclusions**

1048 The use of proteins as therapeutics has significantly improved the treatment of
1049 several diseases, redefining the shape of several medical fields. Therapeutic proteins are
1050 extremely valuable as therapeutics and present a wide range of advantages. Mitigating
1051 the major challenges of the delivery of therapeutic proteins allows a new range of
1052 opportunities. The use of lipid nanoparticles has the potential to overcome the delivery
1053 challenges of therapeutic proteins, allowing their delivery by different administrations
1054 routes. Thus, therapeutic proteins may be loaded into liposomes, SLN and NLC.
1055 However, the use of the NLC for the encapsulation of therapeutic proteins is not well
1056 established yet, and it urges the need to optimize production methods that could not
1057 compromise the protein structure during the encapsulation process. It is foreseen that the
1058 use of lipid nanoparticles to deliver therapeutic proteins will keep on growing the
1059 upcoming years, since several challenges still need to be properly addressed. This review
1060 disclosures those challenges and points out some paths to follow.

1061

1062 **Acknowledgments**

1063 This work was financed by FEDER—Fundo Europeu de Desenvolvimento
1064 Regional funds through the COMPETE 2020—Operational Programme for
1065 Competitiveness and Internationalization (POCI), and by Portuguese funds through
1066 Fundação para a Ciência e a Tecnologia (FCT) in the framework of the project POCI-01-
1067 0145-FEDER-032610—PTDC/MEC-DER/32610/2017. It was also supported by

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1068 national funds from FCT in the scope of the projects UIDB/04326/2020,
1069 UIDP/04326/2020 and LA/P/0101/2020 of the Research Unit Center for Marine
1070 Sciences—CCMAR, and UIDB/04565/2020 and UIDP/04565/2020 of the Research Unit
1071 Institute for Bioengineering and Biosciences—iBB, and LA/P/0140/2020 of the
1072 Associate Laboratory Institute for Health and Bioeconomy—4HB. Cláudia Viegas would
1073 also to thank Fundação para a Ciência e a Tecnologia (FCT) for the PhD fellowship
1074 (2020.08839.BD).

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1516 **Tables**
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1518 **Table 1.** Classification of therapeutic proteins by pharmacologic activity. *Adapted from*
1519 [14,103].
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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

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1531 **Table 2**. Advantages and disadvantages of therapeutic proteins. *Adapted from* [14,15].

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	
Low accumulation in tissues	Difficulty to permeate membranes

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1569 **Table 3.** Excipients used for liposome production. *Adapted from* [65].

Natural phospholipids	Synthetic phospholipids	Unsaturated
Phosphotidylcholine Phosphotidylserine Phosphotidylethanolamine Phosphotidylinositol	1,2- Dilauroyl-sn-Glycero-3-Phosphocoline (DLPC) 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (Sodium Salt) (DOPS) Dipalmitoylphosphotidylcholine Distearoylphosphotidylcholine Dipalmitoylphosphotidylseine Dipalmitoylphosphotidylglycerol	1-Stearoyl-2-Linoleoyl-snGlycero-3-[Phospho-LSerine] (Sodium Salt) Dioleaylphosphotidylcholine
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)		

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1592 **Table 4.** Applications of lipid nanoparticles.
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Nanocarrier	Name	Nanocarrier matrix	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	[104]
	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	[105]
	Lipo-AE vaccine	Phosphatidylserine	Sonication	*Mycobacterium tuberculosis* antigens (Ag85B and ESAT-6)	*Mycobacterium tuberculosis*	[106]
	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*	[107]
	Liposome-based vaccine	Alpha galactosylceramide	Film extrusion method	Palmitoylated synthetic long peptides	Dendritic cells	[108]
	Insulin-Loaded Liposomes	Phosphatidylcholine and cholesterol	Dry thin film hydration method	Insulin	Wound Healing	[109]
	Annexin A5-associated Liposomes	Phosphatidylserine and phosphatidylethanolamine	Thin film hydration method	Ranibizumab and Bevacizumab	Antibodies delivery to the retina	[110]

Nanocarrier	Name	Nanocarrier matrix	Preparation method	Load	Application	Ref.
SLN	Cyclosporine A - loaded lipid nanoparticles	Lipocire DM and Pluronic F-127	Hot homogenization method	Cyclosporine A	Skin-related diseases	[111]
	Erythropoietin - loaded SLN	Glycerin monostearate, span®80/span®60 and tween®80	Double-emulsion solvent evaporation method	Erythropoietin	Neurodegenerative disorders (Alzheimer's disease)	[112]
	Coenzyme Q10 - SLN	Compritol 888 ATO, Poloxamer 188 and Tween 80	High shear homogenization method	Coenzyme Q10	Antioxidants dermal delivery	[113]
	PEG-coated lipid nanoparticles	Miglyol® 812 and tripalmitin	Double emulsion-solvent emulsification method	Peptide salmon calcitonin	Oral administration of peptide drugs	[114]
	Tobramycin-SLN	Stearic acid, Epikuron 200 as and sodium taurocholate	Microemulsion	Tobramycin	Intraocular tobramycin delivery	[115]
NLC	Coenzyme Q10 - NLC	Stearic acid, oleic acid, isopropyl myristate and isopropyl palmitate	High shear homogenization method	Coenzyme Q10	Antioxidants dermal delivery	[116]
	Chitosan coated NLC	Precirol ATO5, Dynasan 114, Miglyol, Tween 80 and Poloxamer 188	Sonication	Model proteins	Brain delivery of proteins by intranasal administration	[73]
	Ovalbumin-NLC	Suppocire NB™, Super refined Soybean oil, lecithin, glycerides and Lipoid S75™	Ultrasonication	Antigen ovalbumin	Development of vaccine formulations	[117]

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1595 **Table 5.** Excipients for SLN and NLC production. *Adapted from* [76].

Excipients	Examples	Properties
Solid lipids	Beeswax	Natural wax with GRAS status and MP of 62-64ºC; 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 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 4–9
	Plantacare® 810	Caprylyl/capryl glucoside, high effective stabilizer for SLN and NLC with HLB of 15-16

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	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 sulphate	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

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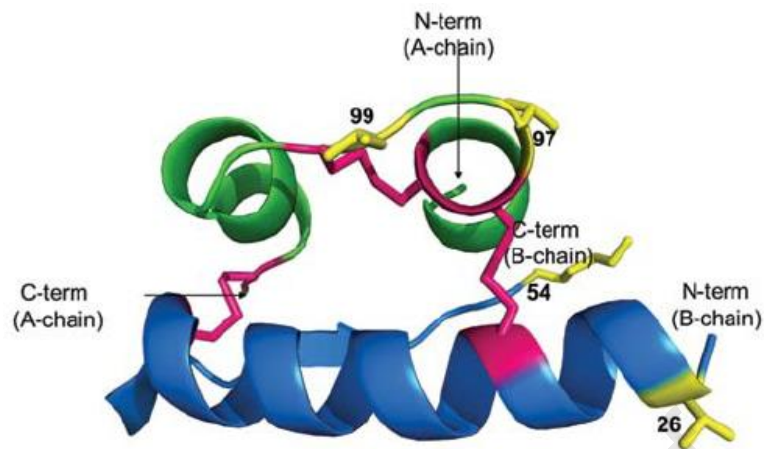

Figure 1. Tridimensional structure of human insulin. A-chain, in green, is covalently connected via disulphide bonds, in pink, to B-Chain, in blue. *Reprinted with permission from* [118].

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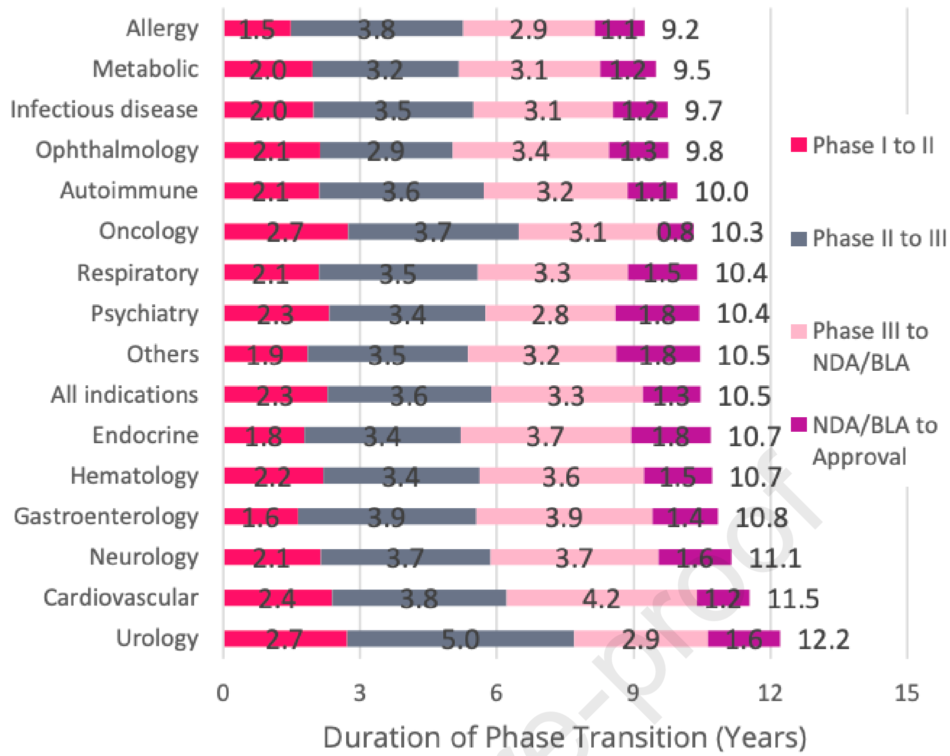

Figure 1. Timeline for product development of therapeutic proteins by disease. These results are based on 6151 successful phase transitions in the 2011–2020 period. *Reprinted from* [22].

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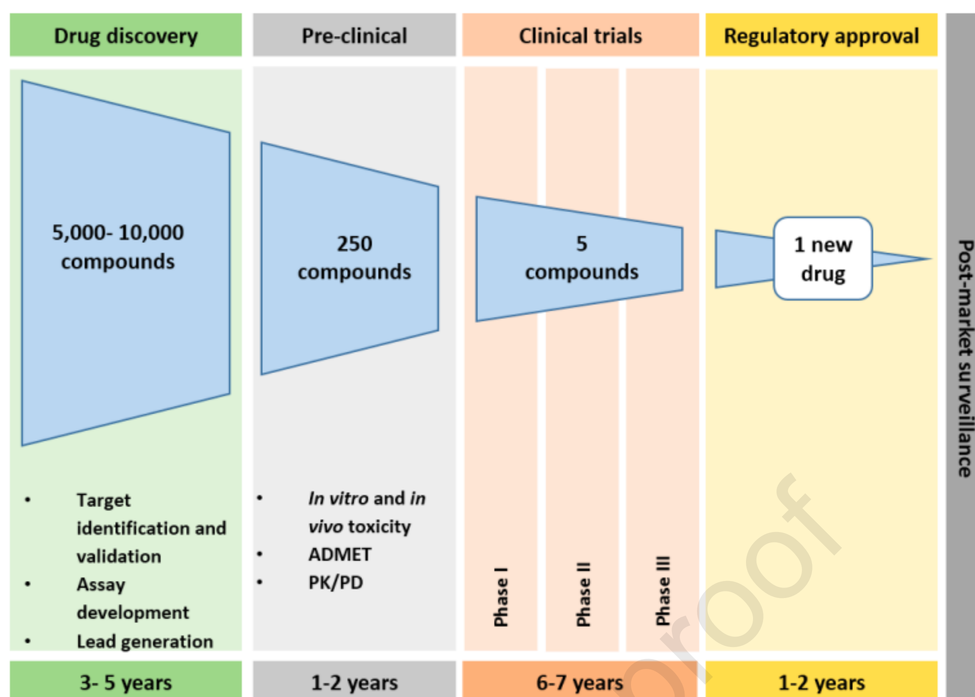

Figure 1. Product development timeline for synthetic drugs. It takes on average approximately 15 years for a synthetic drug to reach the market. *Reprinted from* [119].

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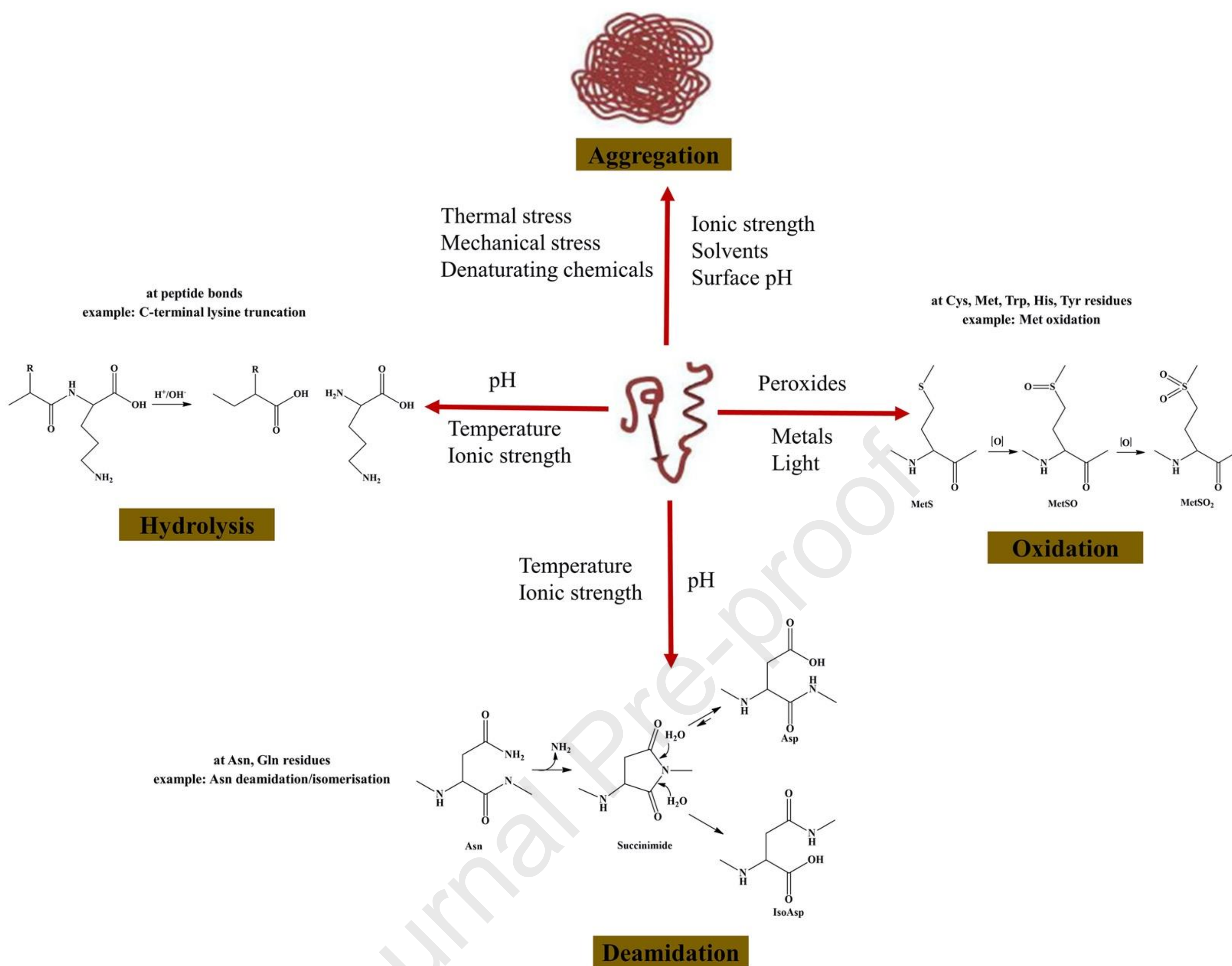

Figure 1. Physical and chemical instability sources of therapeutic proteins. *Reprinted with permission from* [37].

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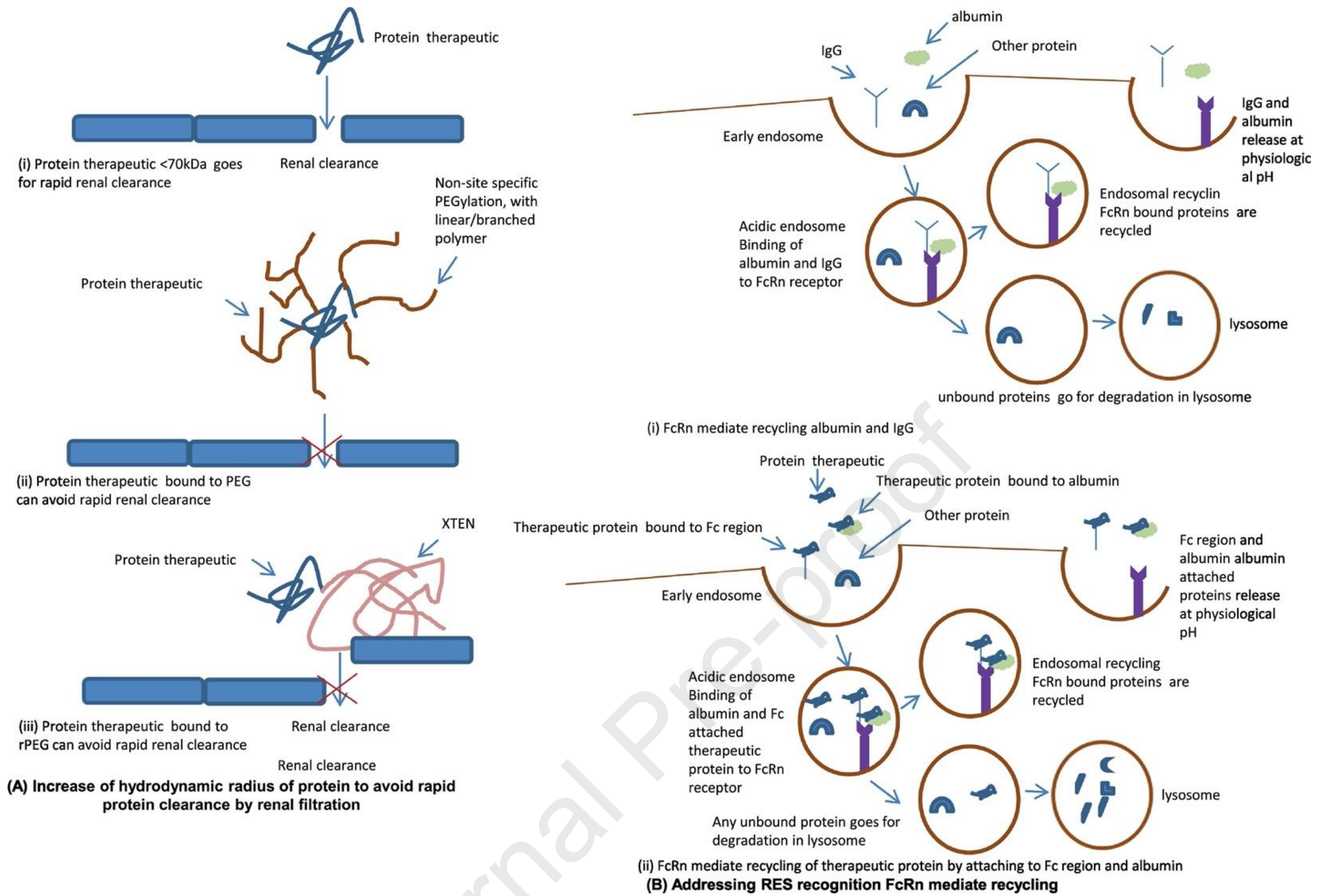

Figure 5. Protein half-life extension by avoidance of rapid renal clearance by pegylation (A) and receptor-mediated recycling (B). *Reprinted with permission from* [17].

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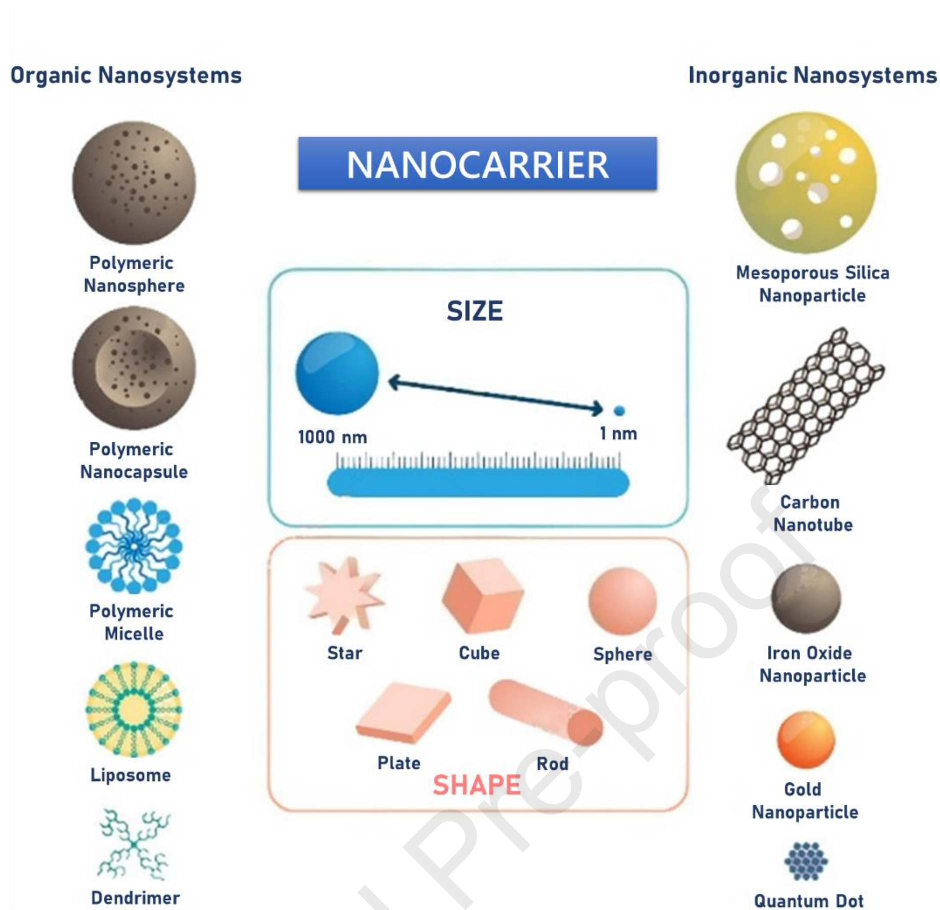

Figure 1. Types, size range and shapes of nanocarriers. *Reprinted from* [120].

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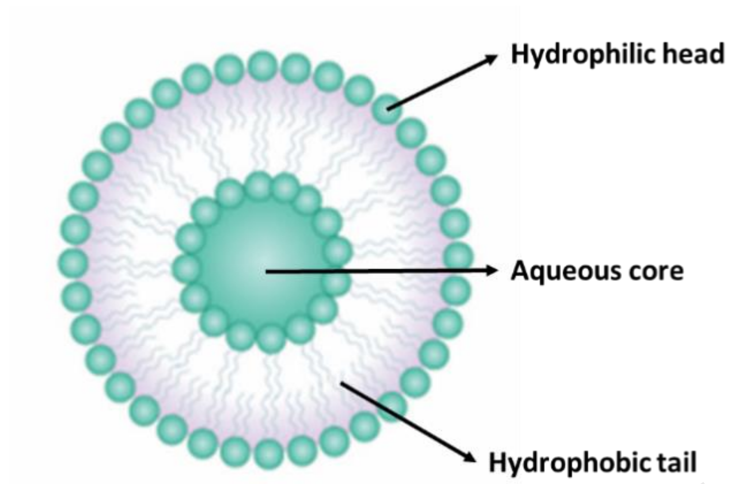

Figure 7. Structure of a typical liposome. Adapted from [66].

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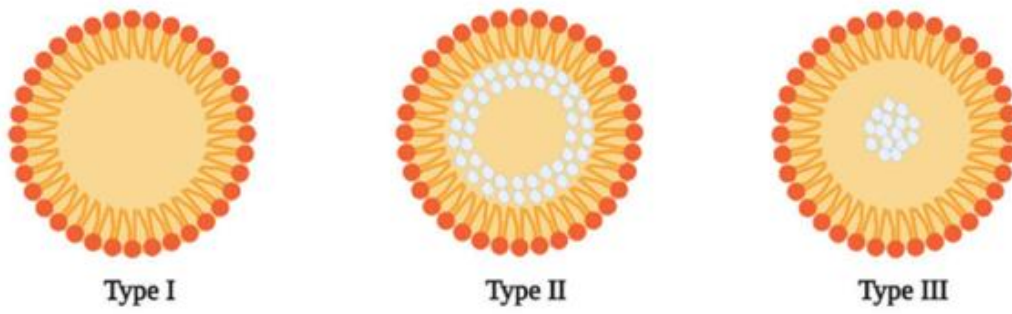

Figure 8. Types of SLN. *Reprinted from* [78].

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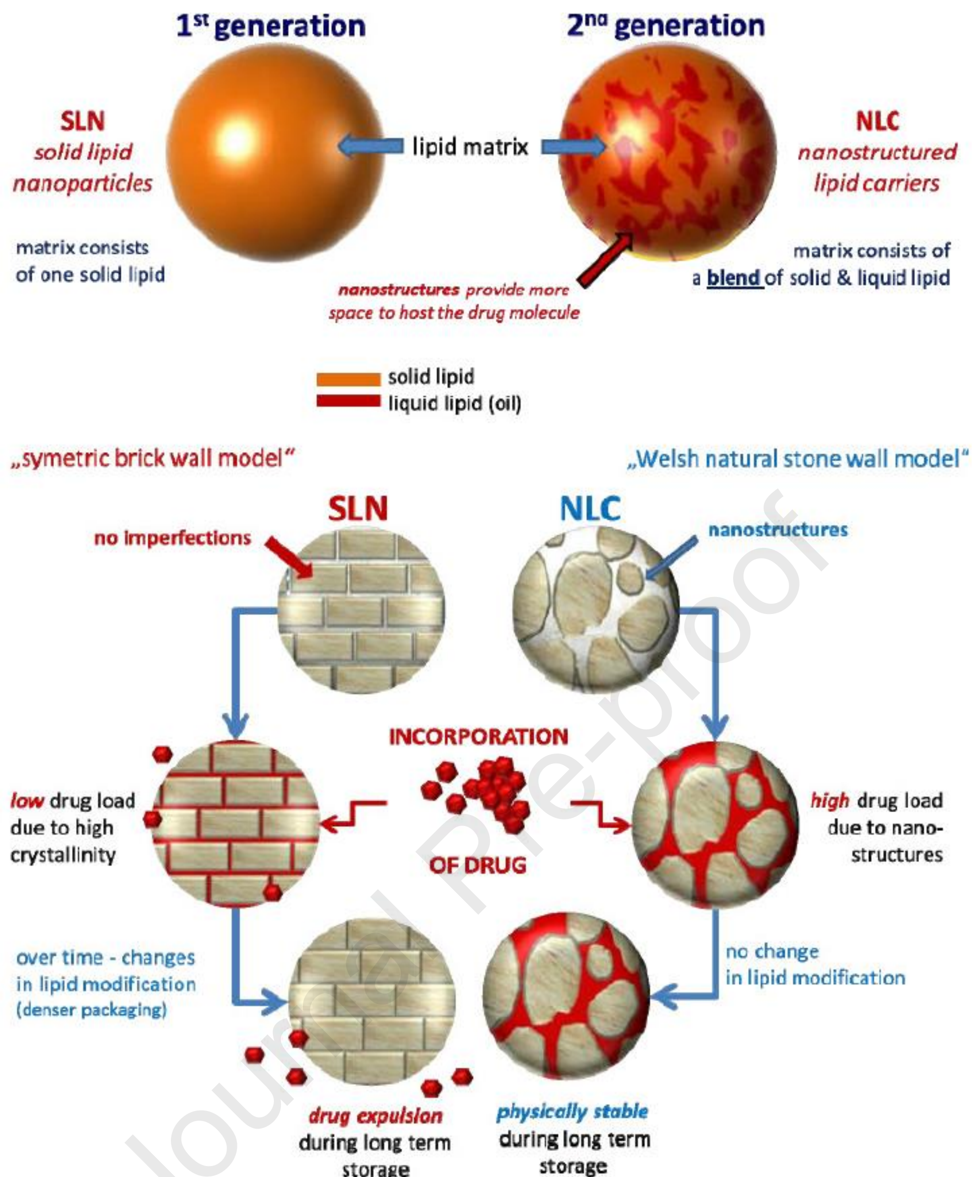

Figure 9. Structural differences between SLN and NLC. *Adapted from* [121].

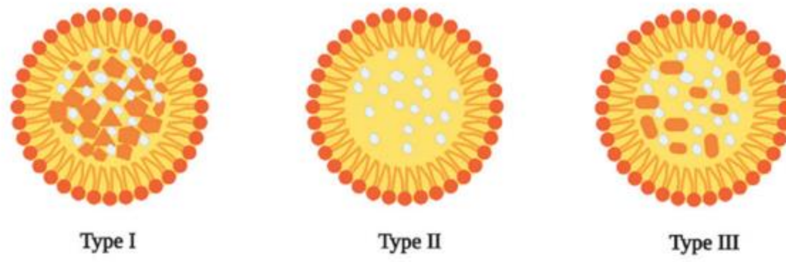

Figure 10. Types of NLC. Reprinted from [78].

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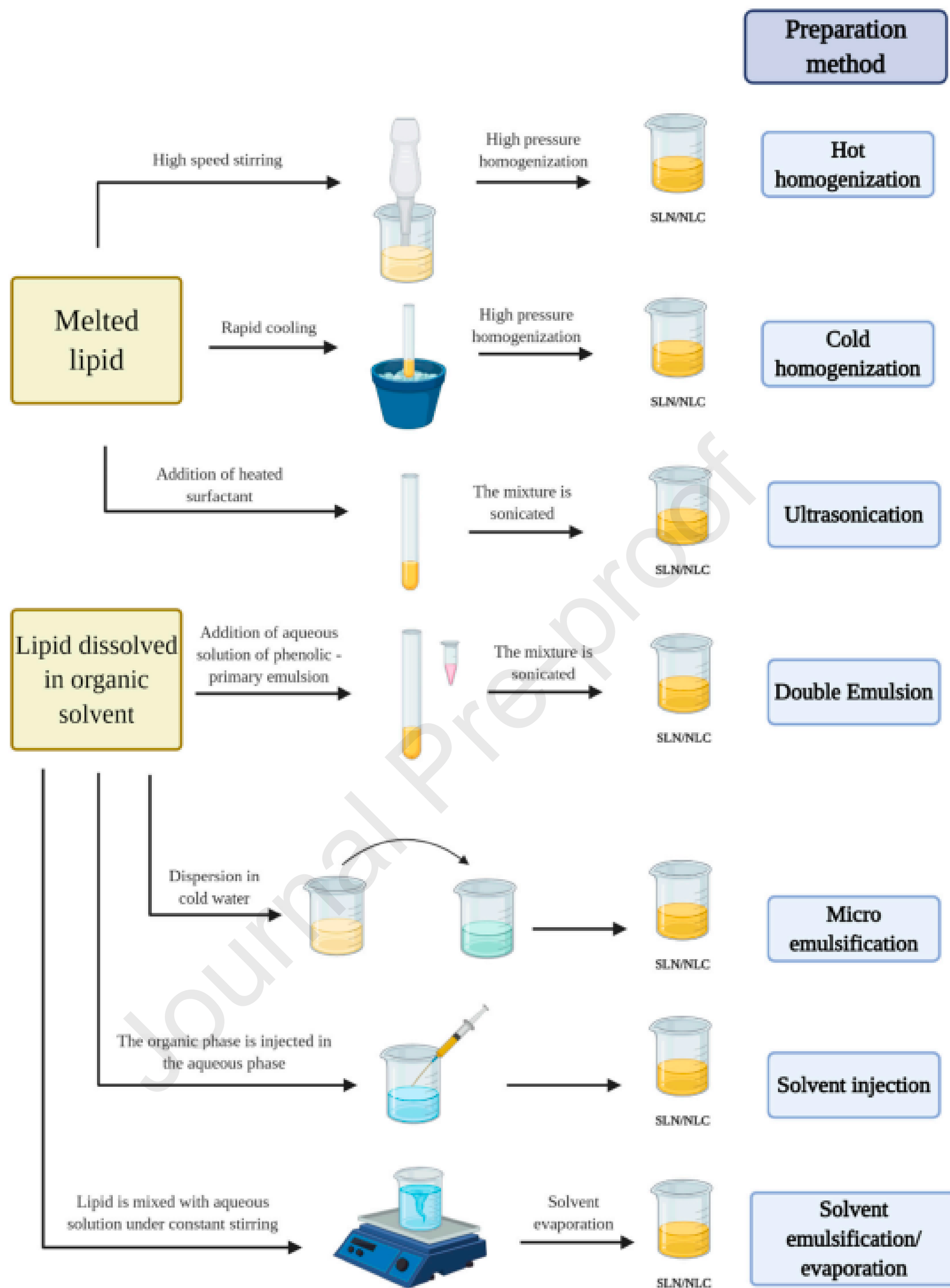

Figure 11. Methods for SLN and NLC production. *Reprinted from* [78].

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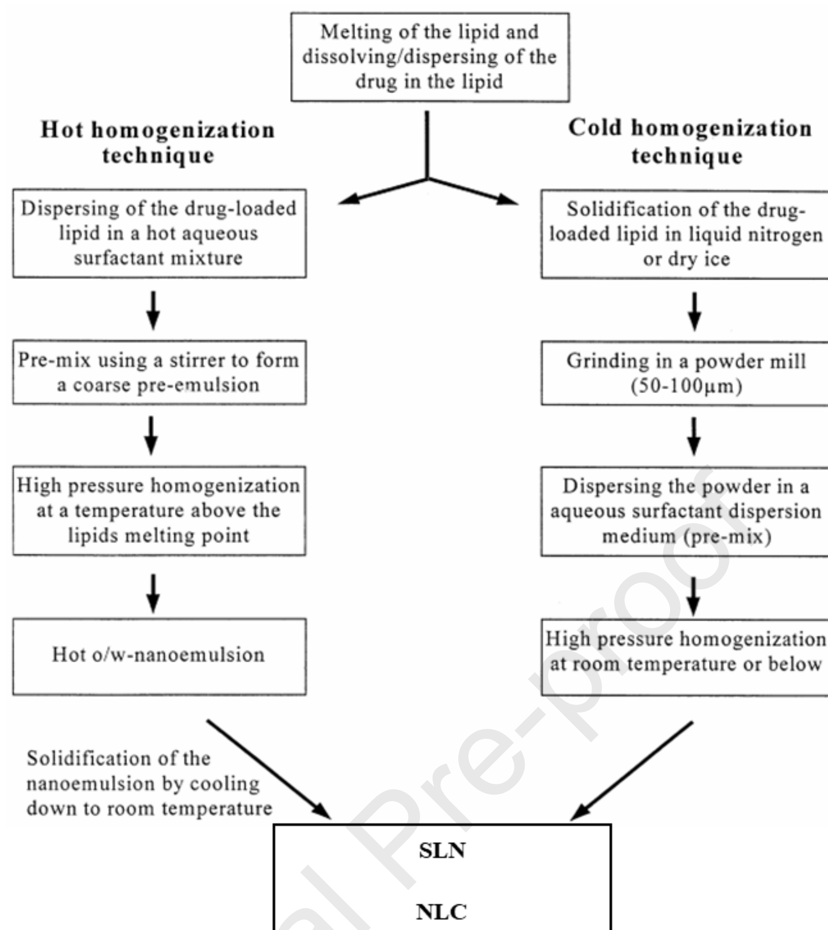

Figure 112. Schematic representation of Hot and Cold Homogenization for SLN and NLC production. *Adapted with permission from* [50].

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Highlights

- The delivery of therapeutic proteins to the body is challenging
- Lipid nanoparticles overcome the challenges of therapeutic proteins delivery
- Different methods are used to tune protein-loaded lipid nanoparticles features
- The maintenance of proteins structure and bioactivity upon encapsulation is crucial
- Further research is needed on developing NLC for therapeutic proteins delivery

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An insight on lipid nanoparticles for therapeutic proteins delivery

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Declarations of interest: none.