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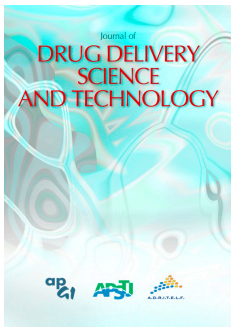

Advances on the formulation of proteins using nanotechnologies

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Advances on the formulation of proteins using nanotechnologies

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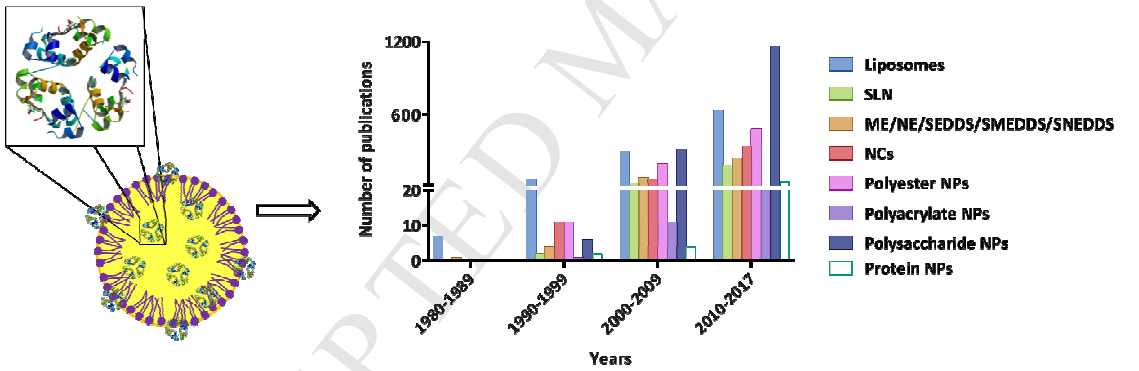
Abstract

Therapeutic proteins and peptides are very attractive from the pharmaceutical point of view due to their high potency and selectivity. Nonetheless, their instability and low bioavailability make their administration through non parenteral routes very difficult, a fact that hampers their efficient exploitation in therapeutics. Since the 70´s, significant amount of research in the area of drug delivery and nanotechnology has been done with the final goal of overcoming those hurdles. In particular, biodegradable and biocompatible lipid and polymer-based nanocarriers have emerged as promising delivery platforms to enable the administration of proteins and peptides. This review provides an overview of the mostly explored nanotechnologies to date intended to produce lipidic and polymeric nanocarriers for protein/peptide delivery. The basic principles of the different techniques are discussed, and the main factors involved in the drug association and release, are analyzed. Finally, a brief overview of the potential applications of these protein/peptide-loaded nanocarriers, highlighting the nanomedicines that have reached the market or the clinical development phase, is provided.

***Keywords**:* protein delivery, peptide delivery, lipid formulation, polymeric formulation, nanocapsule, nanoparticle, liposome, microemulsion

Graphical abstract:



**X-ray structure of Human Recombinant insulin. Image from the RCSB PDB (www.rcsb.org) of PDB ID 5E7W.*

1. Introduction

During the last decades, important efforts have been oriented to the commercialization of therapeutic proteins and peptides. Unfortunately, despite the well-known advantages of these drugs in terms of potency and selectivity, their exploitation is being limited by their instability, restricted bioavailability and intrinsic immunogenicity (specially for high molecular weight proteins) [1]. These draw-backs have stimulated the research in the area of drug delivery and nanotechnology with the final goal of making the administration of these powerful drugs more efficient [2–4].

The possibility of including peptides and proteins in nanovehicles that are able to protect and deliver them at the adequate site has generated increasing expectation during last decades (*Fig. 1*) [5]. Liposomes were the first nanocarriers proposed for protein delivery in the early 70's [6,7]. Meanwhile, Speiser and co-workers investigated the possibility to encapsulate drugs or antigens into polyacrylic nanoparticles using micelle polymerization techniques [8]. A decade later, poly(alkylcyanoacrylate) nanocapsules were proposed as carriers for the oral administration of insulin [9]. Finally, over the 90's Gasco et al. produced for the first time peptide-loaded solid lipid nanoparticles [10–12] and our group pioneered the development of nanoparticles made of PLGA [13], PLA-PEG [14] and chitosan [15] for the delivery of proteins and antigens. As illustrated in *Figure 1*, the interest around the use of all these nanocarriers for protein/peptide delivery has progressively increased in the past decades, being liposomes and polysaccharide-based nanoparticles the ones receiving the greatest attention. Noteworthy, the use of inorganic nanoparticles in the peptide/protein delivery field has grown-up during the past decade, as well. In particular, nanoparticles made of gold, iron oxide [16,17], calcium phosphate and silica likewise carbon nanotubes [18,19] have received a certain attention. However, overall, the tendency has been towards the use of biodegradable and biocompatible biomaterials that can form nanostructures based on friendly and easily scalable techniques. This tendency is expected to change the translational prospective of these delivery vehicles. Indeed, still nowadays the development of efficacious and cost–effective nano-based protein products remains a challenge and this justifies the limited number of protein/peptide-loaded nanoparticulate products in the market [20–22]. The necessity for these nanomedicines to exhibit important quality attributes such as significant drug loading, maintenance of the loaded peptide/protein activity and controlled drug release, are just some examples of the bottlenecks to be overcame [23].

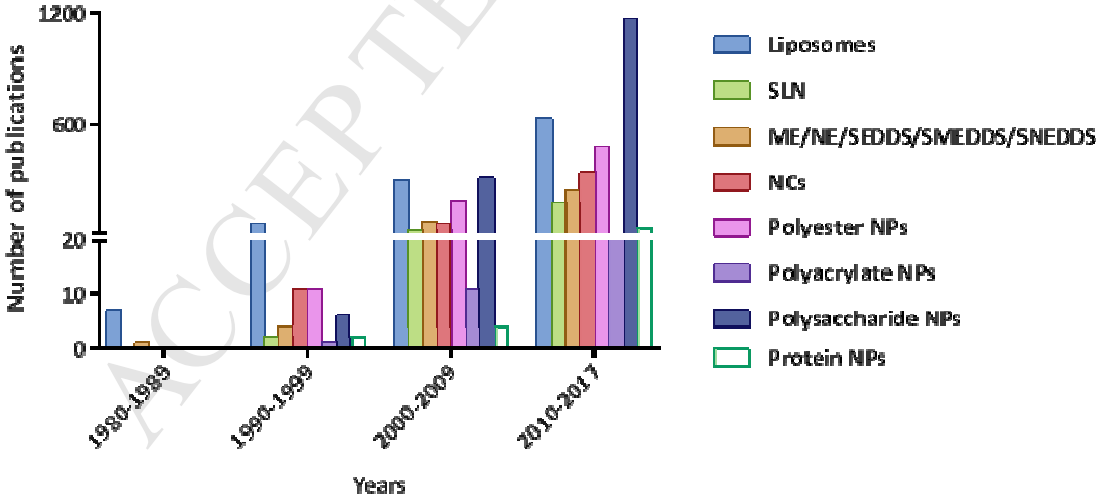


Figure 1. Trend of reported experimental works concerning nanocarriers for peptide/protein delivery from the 70's up to date. Data taken from Scopus (1971–2017) using protein/peptide delivery and the type of system as searching criteria. ME: microemulsion; NCs: nanocapsules; NE: nanoemulsion; NPs: nanoparticles; SEDDS: self-emulsified drug delivery system; SMEDDS: self-microemulsified drug delivery system; SNEDDS: self-nanoemulsified drug delivery system; SLN: solid lipid nanoparticles.

This review aims to analyze the main technologies employed until today to produce lipid and polymer-based nanoparticulate carriers for peptide/protein delivery. Additionally, a brief overview of state of the art of the protein loading, protein structural stability and release properties from these nanocarriers, as well as, their final applications, is discussed. The analysis of the technologies to produce inorganic particles and their characterization was considered to be beyond the scope of this review.

2. Formulation technologies

2.a. Lipid-based nanocarriers

In the last decades, lipid-based nanocarriers (*Fig. 2*) have emerged as potential nanocarriers for macromolecular delivery. This has been mainly due to the absorption enhancing properties of the lipids and the nanocarrier's ability to improve the drug stability. Furthermore, the biocompatible character of these biomaterials and the low cost of the production techniques have increased the interest in these nanocarriers [24]. Despite of this, the inclusion of hydrophilic macromolecules into these systems has been so far limited by their solubility. In order to improve their incorporation into these systems, many innovative strategies, which are summarized below, have been described as promising approaches for the formulation of peptide lipid-based delivery nanosystems (*Fig. 3*).

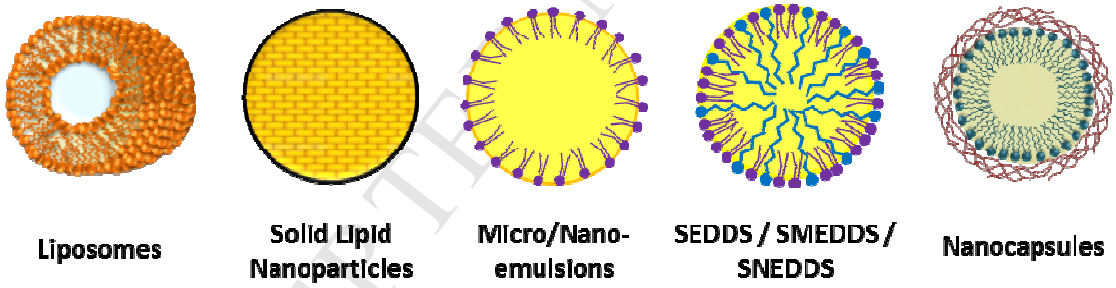


Figure 2. Illustration of the main lipid-based nanosystems explored for protein/peptide delivery. Adapted with permission from [25].

- **Reverse micellization.** This strategy involves the use of amphiphilic molecules able to self-organize as reverse micelles exposing their hydrophobic chains to the exterior and their hydrophilic head groups to the inner part of the structure [26]. This inner cavity facilitates the incorporation of hydrophilic macromolecules prior to its inclusion in the final system [27].

- **Double emulsion method.** This technique consists on the formation of a W/O emulsion in which the hydrophilic drug is confined within its internal aqueous phase prior to its inclusion in the final system [28–30].

- **Hydrophobic ion paring.** This approach has been used to enhance the hydrophobicity of the drug, thereby improving its lipid solubility. It is based on the ionic complexation of a peptide/protein with a molecule, often an amphiphilic compound, with an opposite surface charge [11,31,32] or even with complex structures such as liposomes [33].

- **Hydrophobic – hydrophilic interactions.** This approach involves the dispersion of an aqueous solution of the hydrophilic drug into an amphiphilic compound, followed by the addition of the formed dispersion into the oily phase [34,35].

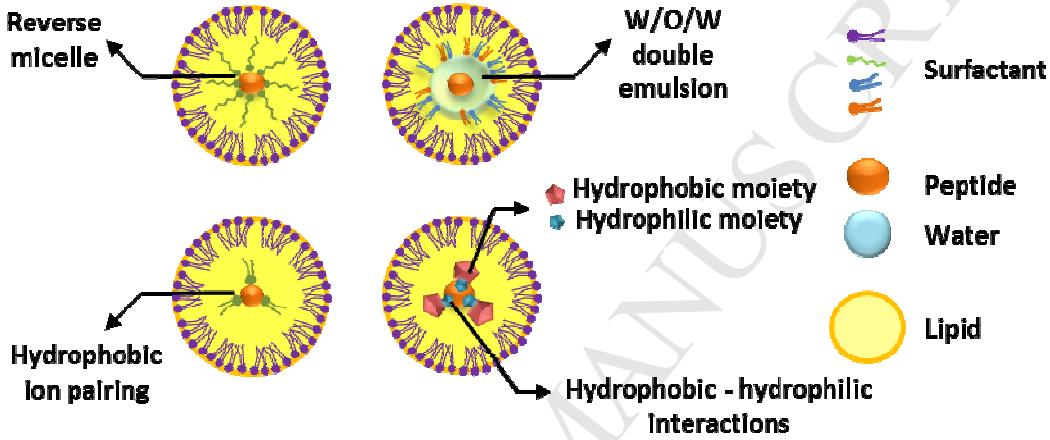


Figure 3. Illustration of the main strategies employed to improve the incorporation of hydrophilic macromolecules into lipid-based delivery nanosystems. Adapted with permission from [25].

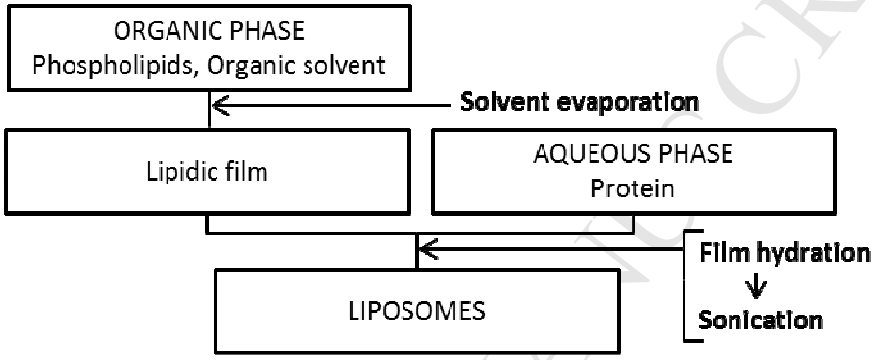
2.a.1. Liposomes

Since their discovery in 1964 [36,37], liposomes have been the most extensively drug delivery vehicles investigated. To date, 13 liposome-based products have been approved for human use by the FDA [25]. Briefly, liposomes are defined as vesicles with an aqueous core in the inner cavity, surrounded by one or more bilayers of amphiphilic phospholipids. Their sizes range from 20 nm (if unilamellar) up to microns (if multilamellar) [38]. Among the wide variety of lipids, those amphiphilic able to self-assembly, such as phospholipids, phosphatidylglycerol derivatives and both saturated and unsaturated fatty acids, are the most commonly used for producing liposomes [25]. Additionally, it is also possible the inclusion of polymers and surfactants into their structure [39–42]. Finally, the use of special lipids has led to the formation of nanostructures named as archeosomes (i.e., diether or tetraether lipids) [43] and niosomes (i.e., polyoxyethylene alkyl ethers) [44], which were supposed to facilitate the entrapment of peptides and proteins [45].

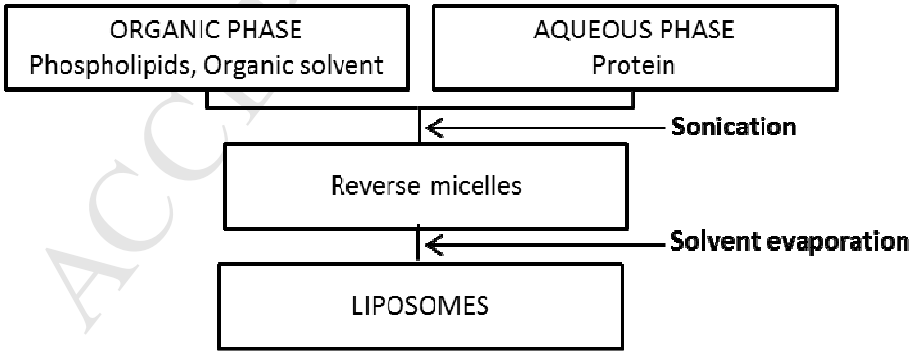
2.a.1.a. Preparation techniques

Overall, the technologies to prepare liposomes are relatively similar. The main difference among the variety of techniques described so far relies on the way of drying the lipids from the organic

202 solvents and rehydrating them in aqueous media [46]. The main liposomes preparation methods
203 used for protein/peptide association are those described below.
204
205 **i) Film hydration**
206 This technique was introduced by Bangham and coworkers to produce liposomes by the first time
207 (*Fig. 4*) [36,37]. This technique involves the dissolution of the phospholipids in an organic solvent,
208 followed by the solvent evaporation and the deposition of the phospholipids forming a lipid film.
209 Then, an aqueous solution containing the protein is added over the lipidic film to hydrate it,
210 usually with the help of sonication, thus leading to the formation of liposomes [47,48].
211



212
213 *Figure 4. Schematic view of the film hydration technique to produce liposomes*
214
215 **ii) Reverse-phase evaporation**
216 This technique simply involves the formation of reverse micelles by mixing an organic solution of
217 the phospholipids with a small volume of an aqueous phase containing the peptide/protein,
218 usually using sonication. The evaporation of the solvent results in the formation of large
219 unilamellar or multilamellar liposomes (*Fig. 5*) [49].
220
221



222
223 *Figure 5. Schematic view of the reverse-phase evaporation technique to produce liposomes*
224
225 **2.a.1.b. Characterization, peptide/protein loading, activity and release profile**
226

- Particle size distribution: generally, a homogenization step is necessary in order to obtain a narrow particle size distribution. The homogenization of the system can be achieved using extrusion [50,51], freeze-thawing [52,53], dehydration-rehydration [54,55], sonication or high pressure. Likewise, the ratio between the different components will influence the final liposomes particle size distribution.

- Peptide/protein loading and activity: liposomes have the ability to encapsulate hydrophilic (in the inner aqueous core), lipophilic (within the lipid bilayer) or amphiphilic drugs (partitioned between the lipid bilayer and the aqueous core) [56]. In general, the driving force for the encapsulation relies on the interaction between the protein/peptide and the lipids and also on the bilayer rigidity. For example, liposomes with insulin association efficiency (AE) values varying from 10 up to 90 % could be obtained by changing the phosphatidylcholine:phosphatidylethanol ratio [57]. To date, a variety of peptides and proteins have been efficiently entrapped into liposomes using the preparation methods disclosed in *Table 1*. Unfortunately, the loading capacity of the resulting formulations has not been described or has been low (< 1 %) [58]. Therefore, the loading capacity could be considered as a limitation of these delivery carriers.

Additionally, the loaded protein must remain active once encapsulated into the liposomes. The sources of peptide/protein instability differ depending on the production method considered, being the film hydration the less stressful for the integrity of the protein, even if sonication could affect its structure [59]. On the other hand, the reverse phase evaporation technique directly exposes the peptide/protein to organic solvents, with the subsequent possibility of suffering denaturation [59,60]. Homogenization, extrusion and freeze thaw cycles can also cause protein denaturation/aggregation in both methods [59]. The integrity of the loaded peptide/protein has been studied using different methods, such as electrophoresis-based techniques (e.g. Western blot, SDS-PAGE, etc.), protein activity (particularly if the encapsulated protein is an enzyme) or directly through *in vivo* experiments [48,57,59,61].

- Peptide/protein release: the physicochemical properties of the phospholipids are known to determine the fluidity of the lipid bilayer and, as a consequence, influence the peptide/protein release profile. In this sense, a more sustained release is obtained when increasing the rigidity of the bilayer by the inclusion of cholesterol or long hydrophobic chains in the liposome [62]. Strategies to control the release of peptides/proteins from liposomes, such as the surface modification with polyethylene glycol (PEG) or other polymers, as well as their inclusion in other nanostructures, have been developed [58,63–66]. For example, a lower insulin release was showed after 4 hours in simulated intestinal fluids from layer-by-layer coated liposomes (20 %) compared to those uncoated (60 %) [48].

In conclusion, both film hydration and reverse phase evaporation methods are suitable for encapsulating peptides/proteins in liposomes, allowing both, good association efficiencies and sustained release profiles. The bilayer rigidity and the electrostatic interactions between the peptide and the liposomes components are the main factors conditioning the loading capacity of

liposomes. Regarding the release behavior, not only the rigidity of the phospholipidic bilayer has an important role in controlling the release, but also factors such as PEGylation, polymer association or their inclusion in other structures can help to obtain sustained release profiles.

Table 1. Examples of peptide/protein-loaded liposomes obtained by the different preparation methods: drug loading and release properties.

Preparation method	Peptide/ Protein	AE (%)	LC (%)	≤1h burst/ cumulative release (time) – pH medium	Ref.
Film hydration	Insulin	88 - 94	n.a.	10 - 40 % / 20 - 60 % (2 h) pH 1.2 10 - 35 % / 20 - 60 % (4 h) pH 6.8 10 - 30 % / 72-96 % (24 h) pH 7.4	[48]
		10 - >90	n.a. (4 mg / 50 mg lipid theor.)	n.a.	[57]
	sCT	91	n.a.	n.a.	[67]
	Leuprolide	17 - 76	n.a.	n.a. / 2 - 16 % (5 h) pH 1.2* / 7.4*	[68]
	BSA	12 - 72	0.1 - 1	20 - 30 % (1 h) pH 7.5*	[58]
Reverse-phase evaporation	Insulin	30 - 83	n.a.	< 20 % / 50 - 95 % (30 h) pH 2* 10 – 30 % / 30 - 80 % (5 h) pH 7.4*	[66]
	Leuprolide	33 - 47	n.a.	No / 40 - 50 % (2 d) pH 7.4*	[69]
		66 - 72	n.a.	n.a.	[70]

AE: association efficiency (100 x associated peptide mass / total peptide mass); BSA: bovine serum albumin; LC: loading capacity (100 x peptide mass / total formulation mass); n.a.: not applicable; Ref.: references; sCT: salmon calcitonin; theor.: theoretical; *Enzyme supplemented.

2.a.2. Solid lipid nanoparticles (SLN)

SLN are nanoparticles made of solid lipids and stabilized by surfactants. These SLN, which were first described in the 90's [12,71], have the peculiarity of being in a solid form at both, room and body temperatures [72]. Among the wide variety of lipids, the long chain triglycerides, fatty acids and phospholipids are the most commonly used for producing SLN [25]. These lipids prevent SLN from rapid degradation, thereby facilitating the control of the drug release [72,73].

2.a.2.a. Preparation techniques

A variety of techniques summarized in *Table 2* have been proposed for the preparation of peptide-loaded SLN. The use of high pressure and temperature, the need of organic solvents, and/or the requirement of sophisticated equipment are the main parameters conditioning the choice of the technique to be used to prepare protein/peptide-loaded SLN.

Table 2. Main characteristics of the most commonly used preparation methods for SLN

Technique	Principle	Stress exposure	Organic solvents	Simplicity
Microemulsi-	Precipitation of the lipidic	Low exposure to T >	No	++

fication / solidification		phase of a ME by dispersing it in cold water	MP of the lipid	necessarily	
HPH	**Hot HPH**	Solidification of a NE previously homogenized under heating by cooling it down	High exposure to T > MP of the lipid; High pressure (100-200 bar)	No	+ (special equipment)
	Cold HPH	Cavitation of a pre-suspension by homogenizing it at ≤RT	Low exposure to T > MP of the lipid; High pressure (100-200 bar)	No	+ (special equipment)
Double emulsion - solvent evaporation		Precipitation of the lipidic phase of a W/O/W emulsion after evaporating its solvent	No	Yes	++
Nanopre-cipitation		Precipitation of a lipid blend by the diffusion of the solvent into an aqueous phase	No	Yes	++
Supercritical fluid technology		Wide variety of methods based on supercritical fluid	Depending on the method and the SCF used (usually CO_2: T_c=31.1 ºC; p_c=73.8 bar)	No necessarily	-- (special equipment)

HPH: high pressure homogenization; ME: microemulsion; MP: melting point; NE: nanoemulsion; p_c: critical pressure; SCF: supercritical fluid; RT: room temperature; T_c: critical temperature.

i) Microemulsification/solidification

This method, originally developed by Gasco and coworkers [10,12] involves two different steps (*Fig. 6*). First, a W/O/W microemulsion containing the hydrophilic peptide in its internal aqueous phase is formed by adding an aqueous solution of the protein with the surfactant and co-surfactant(s) over a melted fatty acid/glyceride mixture (65 - 70 ºC). This W/O microemulsion is then emulsified with a second aqueous phase containing surfactants. The second step consists on the dispersion of this W/O/W microemulsion in cold water (2 - 3 ºC) under mild mechanical stirring [72,74]. The addition of this thermodynamically stable microemulsion to water leads to the precipitation of its lipidic phase forming small particles [10,11]. Alternatively to the double emulsion approach, a primary O/W emulsion containing the hydrophilic peptide as hydrophobic ion pairing in the internal phase can be used [11].

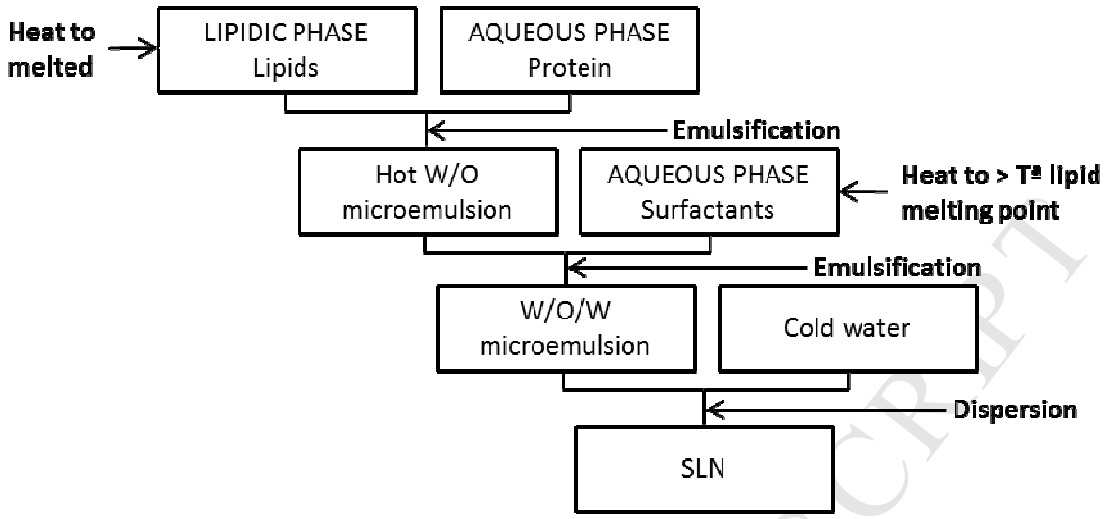


Figure 6. Schematic view of the W/O/W microemulsion-based technique to produce SLN

ii) High pressure homogenization (HPH)

High pressure homogenization is a well-established technique for the preparation of SLN, which can operate either at hot or cold temperature.

- Hot Homogenization. The formation of SLN through this approach involves three different steps (*Fig. 7*). Generally, this method is limited to the encapsulation of hydrophobic peptides/proteins. First, a lipidic phase containing the drug either solubilized or dispersed is melted and dispersed into an aqueous surfactant-containing phase at the same temperature using a high-shear mixing device. This hot pre-emulsion consisting of micrometric droplets is then homogenized under heating until the desirable O/W nanoemulsion is formed. SLN are obtained by simply leaving the system to cool down [75].

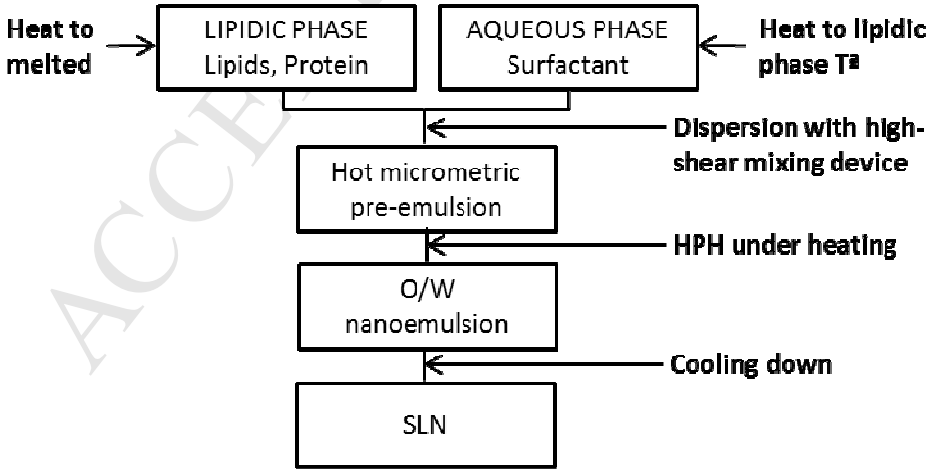


Figure 7. Schematic view of the hot high pressure homogenization (HPH) technique to produce SLN

- Cold homogenization. The cold homogenization process emerged as an alternative to the hot procedure to minimize the drug exposure to high temperatures (*Fig. 8*). However, in this case it is still necessary to heat the lipids above their melting point in order to obtain a liquid phase in which the drug can be solubilized (if hydrophobic) or dispersed as an aqueous solution (if hydrophilic). Then, this melted blend is rapidly solidified, by cooling it down, using dry ice or liquid nitrogen. The obtained solid lipid matrix is then grinded in a powder mill until micrometric particles are formed. In a second step, these micrometric particles are dispersed into an emulsifier solution at or below room temperature to form a pre-suspension. Then, a final high speed homogenization process is carried out in order to break the micrometric particles into SLN [76].

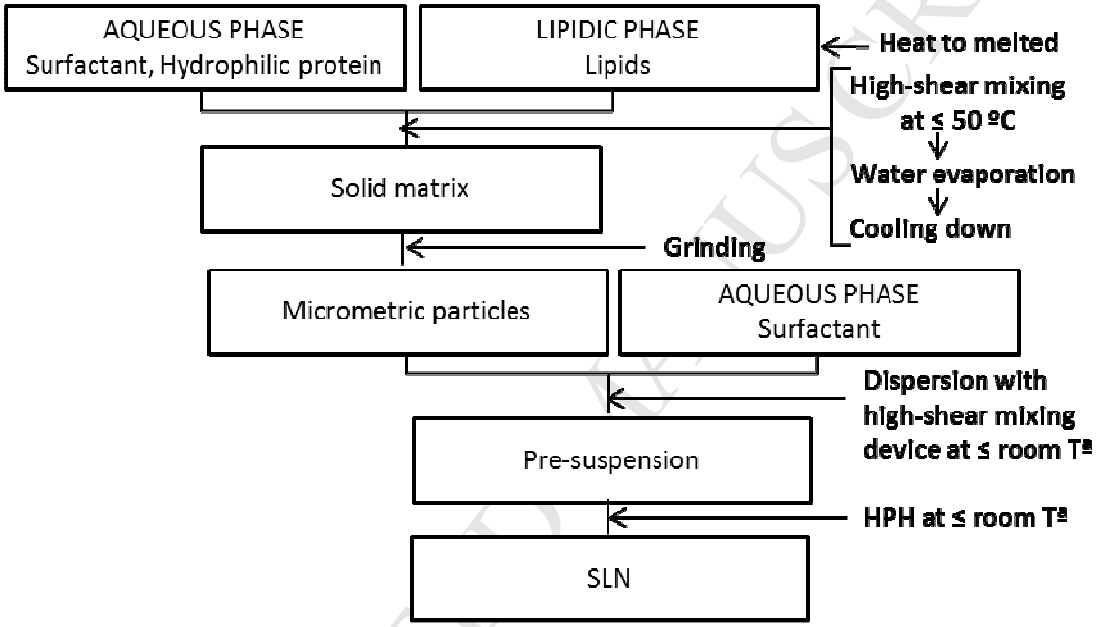


Figure 8. Schematic view of the cold high pressure homogenization (HPH) technique to produce SLN

iii) Double emulsion – solvent evaporation

Sjöström and Bergenståhl were the first describing SLN prepared through its precipitation from O/W emulsions (*Fig. 9*) [77]. To our knowledge, our group was the first adapting this method for the entrapment of hydrophilic protein/peptides by the incorporation of the double emulsion approach [78]. As a first step, an aqueous solution of the protein/peptide drug is emulsified using sonication into an organic phase consisting of lipids and a water-immiscible solvent. Then, this W/O emulsion is emulsified using sonication into an external aqueous phase containing surfactants, leading to the formation of a W/O/W double emulsion. Finally, the organic solvent is removed by evaporation, thereby inducing the precipitation of the lipids in the aqueous phase in the form of nanoparticles [28,29].

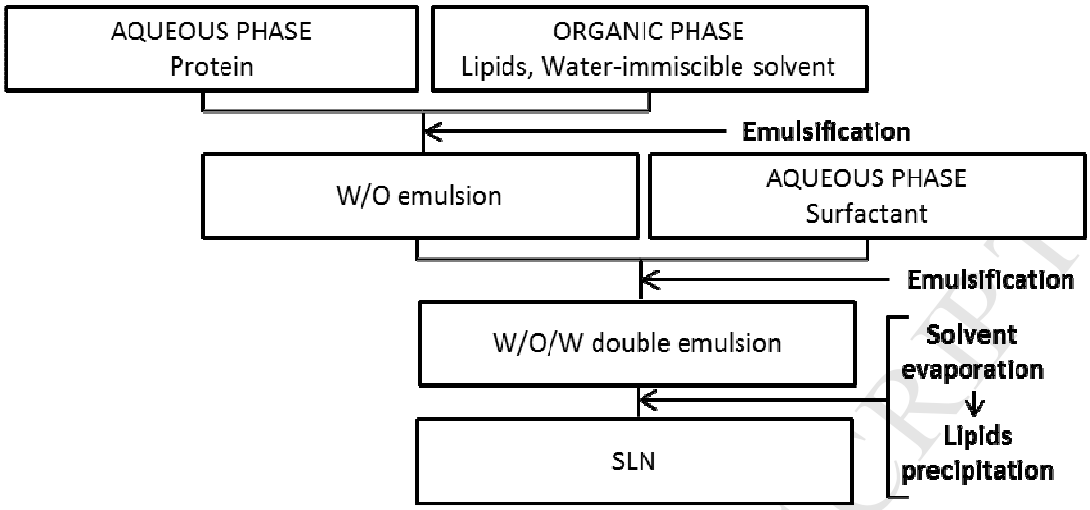


Figure 9. Schematic view of the double emulsion – solvent evaporation technique to produce SLN

iv) Nanoprecipitation

This technique, illustrated in *Fig.10*, is based on the dispersion of a polar solvent containing the peptide/protein and the lipids in a water phase. Due to the immediate diffusion of the solvent the lipids precipitate entrapping the peptide/protein meanwhile. In principle, this technique is adapted for the encapsulation of hydrophobic peptides rather than hydrophilic proteins. However, there is also the possibility to co-dissolve a water-soluble peptide in a water/polar solvent mixture. In some instances the use of high temperature may help to co-dissolve the drug and lipids in the polar solvent [79,80].

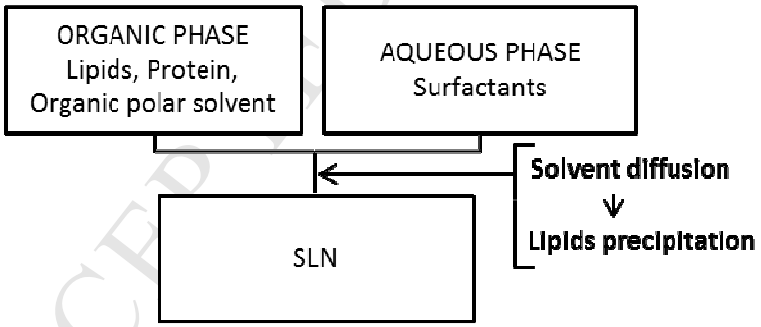


Figure 10. Schematic view of the nanoprecipitation technique to produce SLN

v) Supercritical fluid technology

A new wide range of techniques based on the supercritical fluid technology have recently emerged to produce solvent-free lipid nano- and microcarriers. However, there is very limited information about protein encapsulation through these novel methods. The drug solubility in the supercritical fluid (usually CO_2) is the main parameter to be considered for choosing the most appropriate procedure [81].

2.a.2.b. Characterization, peptide/protein loading, activity and release profile

The physicochemical and pharmaceutical properties of SLN might be influenced by the type of fabrication technique and the formulation variables. An overall analysis of the characteristics of SLN is as follows.

- Particle size distribution: the final size of the SLN is generally influenced by the physicochemical properties (e.g. hydrophilicity, crystallization rate and crystal's shape) and concentration of the lipids in the organic phase. A high viscosity of the lipid phase may hamper its dispersion into the water phase leading to the formation of large particles. The final size is also affected by physical factors, such as the temperature, the homogenization pressure applied during the particle formation, and the number of cycles needed to obtain the formulation. Generally, low polidispersity indexes are obtained when using high stirring rates or high number of homogenization cycles [71,72,82,83].

- Peptide/protein loading and activity: in 1994,Morel et al. attempted for the first time to encapsulate peptides ([D-Trp-6] LHRH and thymopentin) into SLN [10,11] using the microemulsion based technique. Generally, the drug to be encapsulated in SLN is incorporated either directly in the lipidic phase (if hydrophobic) or using a W/O/W double emulsion approach by dissolving it in the internal aqueous phase (if hydrophilic). High AEs (79 - 98 %) and loading capacities (LCs) (6 - 13 %) were attained for hydrophobic peptides such as cyclosporine A using either the microemulsion-based technique [84] or the HPH methods [75]. However, for hydrophilic drugs, SLN have shown limited drug LC, being the solubility of the drug in the lipid matrix the main factor driving the AE [85]. To improve the incorporation of hydrophilic macromolecules into SLN a number of strategies highlighted in section 2.a. have been described. Among them, the double emulsion technique (W/O/W) including or not surfactants in the internal aqueous phase to form reverse micelles has been relatively successfully. This technique was first described by our group for the encapsulation of peptides into SLN, leading to the efficient association (90 %) of salmon calcitonin (sCT) [28,29]. The addition of surfactants, i.e. bile salts, into the internal aqueous phase may lead to the formation of peptide-containing micelles. This approach has led to high AE and LC values for peptides such as sCT [86]. The inclusion of the positively charged sCT and insulin in negatively charged micelles was supposedly the factor favoring the retention of the peptide in the solid core. In fact, some authors showed that the increase of the bile salt concentration and its ratio with the oily phase surfactant had a clear impact on the insulin association efficiency (AE from 20 up to 99 %) [87].

When using the nanoprecipitation method, variables such as the temperature of the dispersed aqueous phase was found to influence the encapsulation of hydrophilic peptides, i.e. gonadorelin (50 % AE at 25 ºC vs. 69 % at 0 ºC). This improvement can be attributed to the rapid solidification of the lipid droplets at low temperature, which would facilitate the entrapment of the peptide [79].

As indicated, the protein/peptide stability is generally influenced by the presence of organic solvents (e.g. double emulsion/solvent evaporation or nanoprecipitation methods), high shear mechanical agitation and pressure (e.g. HPH method), high temperatures (e.g. microemulsification/solidification and HPH methods) or sonication processes (double emulsion/solvent evaporation method) [11,29,30,88]. The protein integrity and activity has been usually analyzed using the same techniques described for liposomes in the previous 2.a.1.b. section (i.e., SDS-PAGE, capillary electrophoresis, enzymatic assays and *in vivo* studies) [11,28–30,76].

- Peptide/protein release: data indicated in *Table 3* highlight the high variability in the release profiles observed for different peptides/proteins entrapped in a variety of SLN [28,78,79]. Although in some works, no burst release was reported, normally there is a variable amount of peptide accumulated at the O/W interface during the production process that is released prematurely [72]. This burst effect and the subsequent release profile has been modulated following specific formulation approaches. In particular, the overall release profile is highly dependent on the SLN composition, since it is mainly governed by the peptide diffusion through the channels, originally present in the matrix, and enlarged in the course of the lipase-mediated lipids degradation [78]. These findings suggest that a selection of the lipidic components is important in order to modulate the protein/peptide release.

It is important to highlight the possibility of an interaction between the peptide/protein and the lipid components and their degradation products. For example, in a work intended to encapsulate leuprolide acetate into SLN using the nanoprecipitation technique, the use of a hydrophobic ion pairing complex between leuprolide and sodium stearate led to a considerable reduction of the burst effect (1 h burst release: 10 % vs. 45 %). Following this initial fast release, the peptide was slowly released for up to 2 days [80]. In another example the sustained release of sCT (40 - 45 % in 6 h) from chitosan-coated SLN produced by the double emulsion-solvent evaporation method was attributed to the high affinity of the positively charged sCT for the negatively charged lipids (lecithin and tripalmitin) [28].

The incorporation of PEG into the lipid matrix has also been proposed as a strategy to modulate the release profile. For example, the release of insulin from SLNs produced by the supercritical fluid technology, could be controlled by incorporating 5 kDa PEG in the lipid mixture as a pore-forming agent [89]. Indeed, the total amount of insulin associated to PEG-containing SLNs was released in 3 days, whereas PEG-free SLNs needed 5 days to deliver their content.

From the results in literature up to date (*Table 3* shows some examples), we can conclude that the release of peptides from SLN is affected by the composition of the lipidic matrix (governing the degradation of the particles) and by the affinity of the peptide/protein towards the formulation components. Normally, the *in vitro* release of the proteins/peptides is prolonged for a few days, however, it could be expected that in an *in vivo* situation the process could be accelerated depending on the degradation rate of the lipidic matrix.

Table 3. Examples of peptide/protein-loaded SLN obtained through the different preparation methods: drug loading and release properties.

Preparation method	Specific strategy	Peptide/ Protein	AE (%)	LC (%)	≤1h burst / cumulative release (time) – pH medium	Ref.
Micro-emulsion-based technique	-	CyA	n.a.	6 - 13	<4 % / <4 % (2 h) pH 7.4	[84]
	Double emulsion	[D-Trp-6] LHRH	90	n.a.	<3 % / 10 % (8 h) pH 6.5	[10]
		Thymo-pentin	2	n.a.	<5 % / 10 % (6 h) pH 6.5	[11]
	Hydrophobic ion pairing	Thymo-pentin	5	n.a.	<5 % / 10 % (6 h) pH 6.5	[11]
Hot HPH	-	CyA	95 - 98	0.5 - 2 theor.	n.a.	[75]
			96	1.9	n.a.	[90]
Cold HPH	-	CyA	79 - 94	0.5 - 2 theor.	n.a.	[75]
		Lysozyme	43 - 59	0.03	n.a.	[76]
Emulsion – solvent evaporation	**Double emulsion**	sCT	31->90	n.a.	<30 % / <45 % (6 h) pH 4	[28, 29]
	Double emulsion / Reverse micellization	sCT	88 - 95	5 - 11	60 - 100 % / 100 % (2 h) pH 6.8*	[86]
		Insulin	76-100	19	0 - 35 % / 60 - 90 % (6 d) pH 7.4	[87]
Nanopreci-pitation	-	Gonado-relin	50 - 69	n.a.	<30 % / <80 % (14 d) pH 6.8	[79]
		Leupro-lide	28	0.3	<45 % / 100 % (2 d) pH 6.8	[80]
	Hydrophobic ion pairing	Leupro-lide	46	0.5	<10 % / 100 % (2 d) pH 6.8	[80]
Supercritical fluid technology	-	Insulin	20 - 80	1 - 4	0 - 17 % / 100 % (6 d) pH 7.4	[89]
			57	2.9	<10 % / 100 % (4 d) pH 7.4	[91]
		rh-GH	48	2.4	<5 % / 100 % (4 d) pH 7.4	[91]

AE: association efficiency (100 x associated peptide mass / total peptide mass); CyA: cyclosporine A; [D-Trp-6] LHRH: agonist triptorelin - luteinizing hormone-releasing hormone; HPH: high pressure homogenization; LC: loading capacity (100 x peptide mass / total formulation mass); n.a.: not applicable; Ref.: references; rh-GH: recombinant human growth hormone; sCT: salmon calcitonin; theor.: theoretical; *Enzyme supplemented.

2.a.3. Microemulsions and Nanoemulsions

Both, water-in-oil (W/O) and oil-in-water (O/W) microemulsions are usually considered as thermodynamically stable and isotropic systems, displaying sizes below 100 nm. The

microemulsion formation has been described as a spontaneous process that occurs after mixing the oil and the water phases containing a certain amount of surfactants, in order to achieve a low interfacial tension between the two phases [92]. Nanoemulsions have also been described as colloidal dispersions that generally display sizes below 200 nm. In contrast with microemulsions, these systems are not isotropic. The nanoemulsion formation requires an external energy input in order to overcome their positive free energy and increase their contact area, leading to the formation of a kinetically stable colloidal dispersion [93]. A special type of emulsions is the one present in the self-emulsifying drug delivery systems (SEDDS) and the self-micro-emulsifying drug delivery systems (SMEDDS) which typically consist of mixtures of oil, surfactant and co-surfactants. Recently, many of these SMEDDS have been classified as self-nanoemulsifying systems (SNEDDS) [94]. Among the wide variety of lipids, the long and medium chain glycerides and fatty acids are the most commonly used for the preparation of self-emulsifying systems, microemulsions and nanoemulsions containing peptides [25]. Medium chain fatty acids are known to improve the peptide solubility and facilitate the emulsification process since their mixture with the aqueous phase is easier.

2.a.3.a. Preparation techniques

A wide variety of methods have been developed to produce micro/nanoemulsions. These techniques can be classified depending on the procedure used to supply energy to the system [95–97], being broadly categorized into the following two groups: i) High-energy processes, which imply the application of mechanical and intensive disruptive forces to the different phases of the system. Special devices are necessary in order to intermingle the oily and the aqueous phases, leading to the formation of nanodroplets (homogenization, microfluidization and ultrasonication) [98–100]; ii) Low-energy processes (spontaneous emulsification and phase inversion), which are based on the spontaneous formation of nanoemulsions either by changing the composition (i.e., ratio surfactant:oil:water, addition of salts, etc.) or the process conditions (i.e., temperature-time profile, stirring, addition speed, etc.) [97,101–104]. Among the wide variety of techniques, those based on the spontaneous emulsification are, so far, the most commonly used for the association of peptides/proteins. This is mainly due to the fact that this method avoids the peptides/proteins being exposed to any temperature or pressure stress.

i) Spontaneous Emulsification.

Through this method, the nanoemulsion is spontaneously formed upon the mixture of the oily and the aqueous phases (*Fig. 11*) [105,106]. The protein/peptide is included in one of them depending on its hydrophilicity or incorporated into the oily phase in a small amount of water. Both phases are immiscible in each other; however, one of the components present in one of them (i.e., an organic solvent, a surfactant) is partially miscible in both. Once the two phases are in contact, a non-equilibrium state is formed, causing the rapid shifting of the miscible component from its original phase into the other. This fact will lead to an increase in the oil-water interfacial area and turbulence, promoting the spontaneous formation of the nanoemulsion [107].

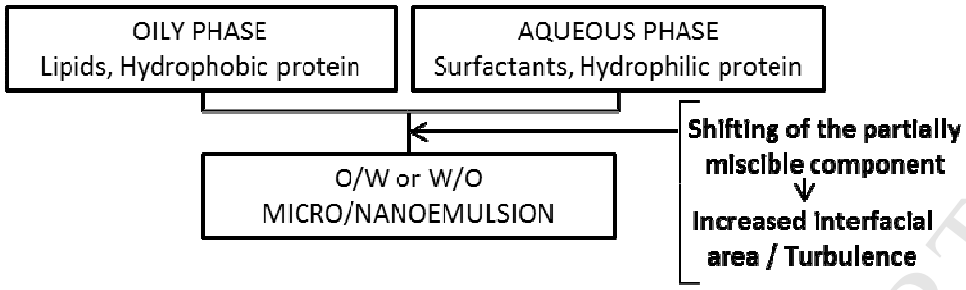


Figure 11. Schematic view of the spontaneous emulsification technique to produce nanoemulsions

2.a.3.b. Characterization, peptide/protein loading, activity and release profile

- Particle size distribution: by selecting appropriately the ingredients and the preparation method, emulsions showing a wide range of sizes, charges and physical properties can be obtained. The final size distribution of the emulsion can be modulated by optimizing its composition (concentration of the components, ratio surfactant:oil:water, interfacial tension, viscosity, emulsifier adsorption kinetics, etc.) and the operating conditions (temperature-time profile, stirring rate, pressure, amplitude of sonication and number of cycles, etc.) [108–111]. The use of ternary phase diagrams is an useful tool to predict the optimum conditions for the formation of the nanoemulsion [112,113].

- Peptide/protein loading and activity: the combination of the spontaneous emulsification technique with several specific strategies, such as, double emulsification, reverse micellization, hydrophobic ion paring or peptide-lipid/surfactant interaction (section 2.a.) has been effective for the loading of hydrophilic peptides (*Table 4*) such as insulin, with AEs higher than 85 % [27,35,114,115]. Among the factors influencing this association, it has been found that small variations in the final pH (from 6.5 to 6.8), may lead to sharp decreases in the AEs from 79 to 30 %. This result was attributed to the different ionization degree of both, the peptide and the polymer at the selected pHs, and their electrostatic and/or hydrophobic interactions [116]. Despite the good association efficiencies achieved, the loading capacity of these systems is usually lower than 1 % [31,117].

After system preparation, the loaded peptide/protein must be able to keep its activity. In fact, there are some operation conditions, i.e. the use of organic solvents and surfactants, which can lead to protein denaturation and/or aggregation. High shear agitations, temperatures or pressures can affect the integrity of the protein, as well [31,118,119]. In this regard, the use of ELISA assays has been reported as an efficient method to understand if the activity of the encapsulated protein is kept. However, in the specific case of micro- and nanoemulsions, direct *in vivo* evaluation of the formulation is the main approach reported to evaluate the efficacy of the loaded therapeutic agent [118,120].

- Peptide/protein release: only a few papers have been published dealing with the mechanism behind the release of the protein/peptide drugs from micro/nanoemulsions. In general, the

release of the drug has been related to its partition between the emulsion and the surrounding medium and also to the alteration/degradation of the lipidic components. For example, when they are orally administrated, their contact with the gastrointestinal fluids can cause a phase inversion or separation of the emulsion phases, that may lead to a premature drug release [120,121]. The conversion of these liquid systems into solid forms through freeze drying, spray drying, melt granulation, melt extrusion or adsorption over solid carriers has been proposed as a way to overcome the colloidal instability of these systems [122]. Further improvements of this technology in order to optimize the delivery of hydrophilic drugs from self-emulsifying systems are still needed. However, for lipophilic peptides, some formulations, such as Neoral® (SMEDDS containing cyclosporine) have already been marketed [1].

Table 4. Examples of peptide/protein-loaded micro/nanoemulsions and SEDDS/SMEDDS/SNEDDS obtained through the spontaneous emulsification method: drug loading and release properties.

System	Specific strategy	Peptide/ Protein	AE (%)	LC (%)	≤1h burst/ cumulative release (time) – pH medium	Ref.
O/W	**-**	sCT	> 90	n.a.	n.a.	[119, 123]
		Pliti-depsin	95-98	0.54	n.a.	[124]
W/O	**-**	TAT	97	0.006 TAMRA-TAT	90 % (1 h) pH 6.8*	[120]
		rhPTH1-34	83	n.a. (45 mg/mL)	100 % (50 min) pH 8* 65 % / 80 % (2 h) pH 2*	[125]
		Insulin + aprotinin	97	0.1 (30 IU/g)	0 % (1 h) pH 1.2*	[118]
	Reverse micelles	Insulin	> 85	n.a. (2.2 % w/v theor.)	n.a.	[27]
	Hydrophobic ion pairing	Insulin	30 - 79 (complex-ation)	n.a.	<10 % (1 h) pH 1.2*	[116]
W/O/W	**Double emulsion**	Insulin	96 - 97	n.a. (18 IU/g)	0 - 80 % / 0 - 80 % (1.5 h) pH 7	[114]
		Insulin + aprotinin	88 - 97	0.075	20 - 30 % / 20 - 30 % (2 h) pH 7	[115]
		sCT + aprotinin	n.a.	n.a. (400 IU/g theor.)	90 % / 100 % (2 h) pH 6.4/1.2* 80 % / 80 % (2 h) pH 7.5*	[126]
SMEDDS SNEDDS	**Hydrophobic ion pairing**	Insulin	64 - 71	0.3-1.1	15 % / 30 % (8 h) pH 7.4	[117]
		Leupro-relin	59 (complex-ation)	0.4 theor. complex	<20 % / 40 % of complex (30 h) pH 6.8	[31]
	Hydrophilic-hydrophobic	Insulin	85 - 99	n.a.	1 % / 14 % (24 h) pH 7.4	[35]

interactions

AE: association efficiency (100 x associated peptide mass / total peptide mass); LC: loading capacity (100 x peptide mass / total formulation mass); n.a.: not applicable; Ref.: references; rhPTH1-34: recombinant 1-34 N-terminal fragment of endogenous human parathyroid hormone; sCT: salmon calcitonin; TAMRA: tetramethylrhodamine; TAT: HIV transactivator of transcription; theor.: theoretical; *Enzyme supplemented.

2.a.4. Nanocapsules

Nanocapsules are core-shell structured drug delivery carriers. They consist of an oily core which is stabilized by surfactants and it is surrounded by one or more polymer shells [127]. Both, core and outer shell layers, play a crucial role in the outcome of the formulation: whereas the core usually works as a drug reservoir, the polymer coating helps the associated drug to overcome biological barriers and modulate its release profile. Among the wide variety of lipids, the long chain fatty acids and the medium chain glycerides (mono-, di- and tri-), both showing penetration enhancer properties, are the most commonly used for producing nanocapsules [25].

2.a.4.a. Preparation techniques

The preparation of nanocapsules involves the emulsification of an oily phase into an aqueous phase. The polymer forming the shell can be incorporated into the organic phase or the aqueous phase [128,129]. Additionally, two different polymers can be incorporated one in each phase [130,131]. The shell is formed due to its precipitation at the interphase or to an ionic interaction between the oily core and the polymer. In a different situation, i.e. poly(alkylcyanoacrylates), the polymer shell is formed due to an interfacial polymerization process [9,132]. The main factors driving the choice of the appropriate nanocapsules production technique are the nature of the polymer as well as that of the peptide/protein to be encapsulated (*Table 5)*.

Table 5. Main characteristics of the most commonly used preparation methods for nanocapsules

Technique		Principle	Stress Exposure	Organic solvents	Simplicity
***"In situ"* polymerization / Interfacial polymerization**	**Oily core nanocapsules**	Monomers polymerization "*in situ*" at the interface of an emulsion	Undesirable reactions drug-monomers / Vigorous stirring	Yes	+
	Aqueous core nanocapsules			No necessarily	
Polymer precipitation/ deposition	**Solvent displacement**	Solvent diffusion to the aqueous phase and polymer precipitation/deposition	Moderate stirring	Yes	++
	Self-emulsification	Surfactant shifting from the oily to the aqueous phase and polymer deposition	High surfactant concentration / Moderate stirring	No	++

i) *"In situ" p*olymerization.

In this method, which is also named as interfacial polymerization, the polymer formation occurs "in situ" at the interface of an emulsion through a fast polymerization among reactive monomers. Due to their rapid and easy polymerization, alkylcyanoacrylates have been the monomers of choice for this purpose [133,134]. Unfortunately, the potential reaction between the drug and the reactive monomers during the process constitutes a limitation of this approach [135].

- Interfacial polymerization in oily core nanocapsules. In this case, the organic phase is composed by the peptide/protein, the oil, the monomers and an organic solvent. The solvent needs to be water-miscible in order to promote its diffusion towards the aqueous phase, allowing the spontaneous formation of nanometric oily droplets [136]. The organic phase is usually injected into the aqueous phase, which contains at least a hydrophilic surfactant. This process is usually performed under vigorous stirring, leading to the instantaneous formation of the nanocapsules (*Fig. 12*). An additional final step to remove the organic solvents can be performed [9,134].

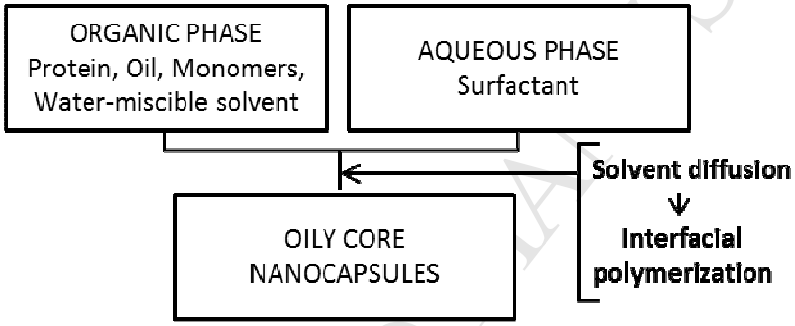


Figure 12. Schematic view of the interfacial polymerization technique to produce oily core nanocapsules

- Interfacial polymerization in aqueous core nanocapsules. In this method, the aqueous phase, which contains the protein/peptide, water and sometimes water-miscible solvent, is emulsified into an organic phase consisting of an oil and a lipophilic surfactant using sonication or vigorous stirring. Once the W/O emulsion is formed, the monomers are added under mechanical stirring. This last step, triggers the polymerization at the W/O interface and leads to a final system consisting of aqueous core nanocapsules dispersed in oil (*Fig. 13*) [137,138]. The nanocapsules are finally isolated by ultracentrifugation followed by their resuspension in water [139,140].

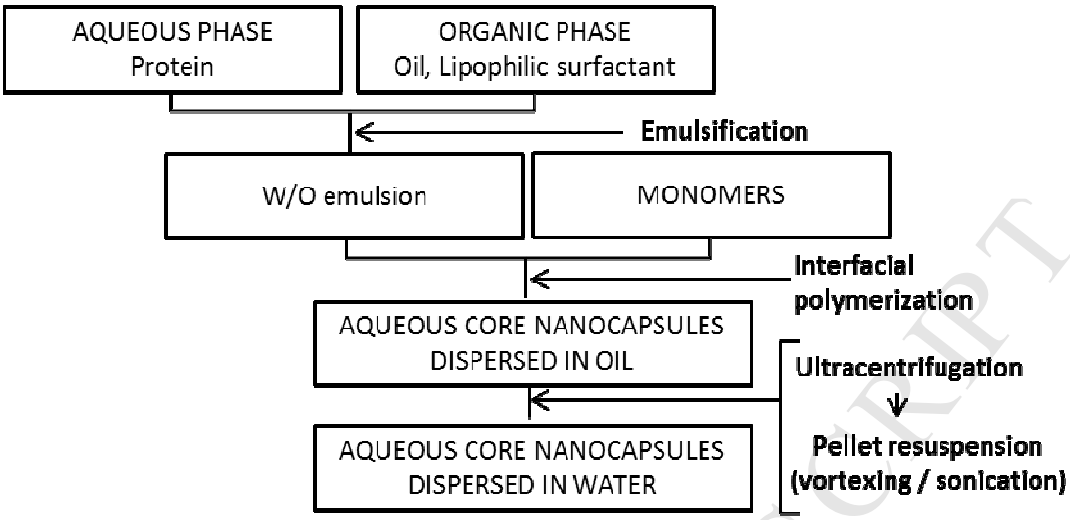


Figure 13. Schematic view of the interfacial polymerization technique to produce aqueous core nanocapsules

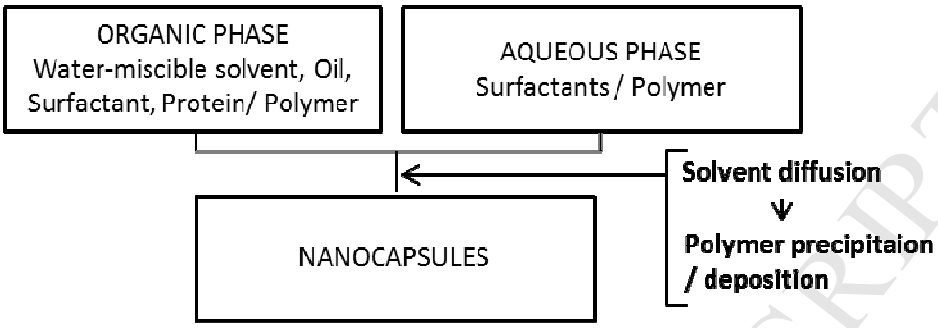
ii) Polymer precipitation/deposition

Contrarily to the "*in situ*" polymerization, the use of preformed polymers allows a good control of the final polymer molecular weight, avoiding undesirable reactions between the drug and monomers. In this case, the polymer coating can be formed by either polymer precipitation or polymer deposition/interaction.

- Polymer precipitation. This technology was first reported by Fessi and coworkers [141,142]. This method involves the use of an organic polar phase containing a lipophilic surfactant, an oil, and the polymer, and an aqueous phase, that may contain hydrophilic surfactants. The usual procedure can be summarized as follows (*Fig. 14*): the organic phase is added dropwise over the aqueous phase under moderate stirring leading to the instantaneous diffusion of the water-miscible solvent from the lipophilic solution to the aqueous phase. As a consequence, the polymer precipitates at the interface of the formed oily droplets, stabilizing them. In a final step, solvents can be removed by evaporation under vacuum [128,130,142].

- Polymer deposition/interaction. Alternatively, nanocapsules can be produced using water soluble polymers according to a deposition/interaction technique. In this case, the polymer shell is formed due to its ionic interaction with the lipophilic components of the oily core. This interaction may occur during the solvent displacement process or after the incubation of the preformed nanoemulsion with the water-soluble polymer [123,129,143,144]. Additionally, the possibility of obtaining multi-layer nanocapsules has been reported. This layer by layer approach is based on the adsorption of different polymeric layers onto a colloidal template. The addition of each polymeric layer should invert the overall charge of the system in all the absorption steps [127]. Our group has reported the possibility of obtaining protein-loaded nanocapsules by triggering the polymer deposition by a **self-emulsification** method avoiding the use of organic solvents. The principle of this technique is the same described in section 2.a.3.a. for the spontaneous formation of

644 micro/nanoemulsions (*Fig. 11*), including, additionally, a water-soluble polymer into the aqueous
645 phase [145] or in a subsequent incubation step [146,147].
646



647
648 *Figure 14. Schematic view of the solvent displacement - polymer precipitation/deposition technique to produce*
649 *nanocapsules*
650
651 **2.a.4.b. Characterization, peptide/protein loading, activity and release profile**
652
653 **- Particle size distribution:** the main factors affecting the final particle size distribution of
654 nanocapsules are the ratio and the mixing conditions between the two phases, as well as the
655 physicochemical properties and concentration of the different components
656 [119,128,129,148,149]. Overall, nanocapsules have been produced so far with a size between 30
657 and 400 nm.
658
659 **- Peptide/protein loading and activity:** Couvreur and coworkers were the first reporting the
660 possibility of using nanocapsules as delivery vehicles for proteins [9]. Since their contribution
661 through the encapsulation of insulin in poly(alkylcyanoacrylate) nanocapsules, several authors
662 have demonstrated the capability of nanocapsules to entrap different peptides/proteins (*Table 6*).
663 Despite the high AEs attained, the LC values reported so far are below 2 %, which is usually due to
664 the hard solubilization of hydrophilic peptides into the lipidic phase and their tendency to diffuse
665 to the outer aqueous phase [139]. When nanocapsules are obtained by interfacial polymerization,
666 the monomer concentration has been proved to be one of the main factors influencing the
667 peptide association efficiency [150]. The pH of the peptide solution has also been shown to
668 influence the AE of peptides to PACA nanocapsules. This effect is attributed to the influence of the
669 pH on the polymerization rate of the polymer [148].
670
671 Our group has also shown the possibility to attach proteins to preformed polymer nanocapsules.
672 For example, we have efficiently associated the recombinant hepatitis B surface antigen (rHBsAg)
673 onto preformed chitosan nanocapsules. In this situation the attachment of the protein was found
674 to be dependent on both protein and nanocapsules concentration and the mechanism of
675 attachment was based on ionic/hydrophobic interactions [151–153].
676
677 Different formulation parameters could influence the peptide/protein structure. With "*in situ*"
678 polymerization the drug could work as a monomer during the polymerization procedure, being

denatured and losing its activity. However, ethanol can be used to preserve the peptide/protein structure [154]. Furthermore, in all the techniques described above for nanocapsules production, the presence of organic solvents and surfactants, as well as the vigorous stirring, could also affect the structure of the encapsulated peptide/protein [139,143,148]. Electrophoresis-based techniques (e.g. native SDS-PAGE), HPLC-based methods or circular dichroism have been reported to study the structural stability of nanoencapsulated peptides/proteins [139,148,154]. However, in the majority of the works, the activity of the encapsulated drug was evaluated after its *in vivo* administration [128,143,155].

- Peptide/protein release: the mechanism driving the release of peptides/proteins entrapped into nanocapsules has been defined as a combination of two main processes: the partition of the drug between the nanocarrier and the external release medium and the degradation of the polymer shell and the lipid core. Both processes can be affected by different factors, such as the pH of the release medium, the nature of the lipidic cores, the type and molecular weight of the polymer, as well as the thickness of the polymer shell [138,143,149,156]. BSA cumulative releases ranging from 35 % up to 90 % were reported for poly(butylcyanoacrylate) nanocapsules after 8 h in release media with different pHs (from 2.5 to 8.5) and different profiles were showed when poly(butylcyanoacrylate) of 4, 7 or 10 kDa was used. Likewise, the loading and the molecular weight confer the protein with different diffusion capacities and specific interactions with the components of the system. High loadings increase the protein gradient between the nanocapsule core and the outer phase, and proteins with high molecular weights diffuse more slowly through the polymeric wall [139]. On the other hand, when the protein is attached to the polymer shell, the mechanism of release is based on its disassociation [157] and this process is normally dependent on the pH and ionic strength of the release medium.

From the results in literature up to date, we can conclude that the solvent displacement technique is the most advantageous for encapsulating hydrophilic peptides in nanocapsules. Apart from its simplicity, and the possibility of controlling the exact molecular weight of the polymer and avoiding undesirable cross-reactions, high association efficiencies can be attained.

Table 6. Examples of peptide/protein-loaded nanocapsules obtained by the different preparation methods: drug loading and release properties.

Preparation method	Peptide / Protein	AE (%)	LC (%)	≤1h burst / cumulative release (time) – pH medium	Ref.
Interfacial polymerization (Oily / Aqueous core)	Insulin	55 - 98	n.a.	n.a.	[9,158, 159]
		90	n.a. (0.45 mg/mL)	10 % / 13 % (5 h) pH 7.4/1-2* 77 % / 80 % (5 h) pH 6-7*	[154, 160, 161]
		57 - 95	n.a.	n.a.	[148, 162]
		100	n.a.	n.a.	[163]
	Human	35 - 79	n.a. (0.0067 -	40 - 60 % (20 min) pH 7.4*	[164]

	calcitonin		0.67 mg/mL theor.)		
	Octreo-tide	60	n.a.	n.a.	[132]
	Insulin	63 - 97	n.a.	25 - 50 % / 50 - 70 % (6 h) pH6.8 No / <5 % (2 h) pH 1.2	[137, 138]
		12 - 52	0.6 - 1.8	35 - 90 % / 80-100 % (6h) pH 6.8	[156, 165]
	OVA	8 - 95	n.a.	n.a.	[150]
	BSA	90	n.a.	n.a.	[166]
		n.a.	1 - 4	15 - 60 % / 60 - 90% (9 h) pH 7.4 15 - 50 % / 40 – 80 % (8 h) pH 2.5/5.5/7.2/8.5	[139]
	D-Lys6-GnRH	95 - 99	n.a.	No / <11 % (5 d) pH 7.4 No / <5 % (6 h) pH 6.8*/1.2* <60 % / <60 % (4 h) pH 7.4*	[167, 168]
Polymer precipitation	CyA	99	n.a.	75 % (5 min) pH 7.4	[128]
Polymer deposition/ interaction	Insulin	51 - 62	0.8 - 1	20 - 30 % / 20 - 40 % (6 h) pH 1.2/6.8/7.1 40 - 70 % / 65-80 % (2h) pH 6.8*	[129]
		99	n.a. (0.5 mg/mL)	n.a.	[154]
	Elipsi-depsin	46 - 54	n.a. (0.25-1.6 mg/mL theor.)	n.a. / 10 % (4 h) pH 6.8	[155]
	Pliti-depsin	98 - 99	0.54	60 - 70 % / 60-70% (24 h) pH 7.4	[124]
	sCT	44 - 60	n.a.	10 – 20 % / 10-20 % (6 h) pH 4	[123, 143, 144]
	rHBsAg	55 - 83	n.a	n.a	[151–153]
	CyA	95	5	n.a.	[146]

AE: association efficiency (100 x associated peptide mass / total peptide mass); BSA: bovine serum albumin; CyA: cyclosporine A; D-Lys6-GnRH: agonist gonadotropin releasing hormone; LC: loading capacity (100 x peptide mass / total formulation mass; n.a.: not applicable; OVA: ovalbumin; Ref.: references; rHBsAg: recombinant hepatitis B surface antigen; sCT: salmon calcitonin; theor.: theoretical; *Enzyme supplemented.

2.b. Polymer-based nanocarriers

Polymer-based nanocarriers (*Fig. 15*) have been widely used for the delivery of proteins and peptides. Both, hydrophobic (e.g. poly(lactide-co-glycolide) and hydrophilic (e.g. polysaccharides) polymers have been employed during the last years for the encapsulation of peptides and proteins

with some promising results. Here, a brief overview of the polymers and techniques used to produce protein/peptide-loaded nanoparticles is given.

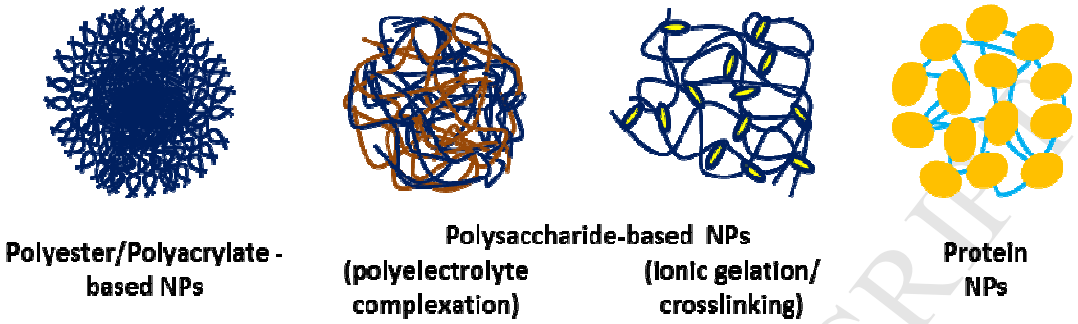


Figure 15. Illustration of the main polymer-based nanoparticles (NPs) used for protein/peptide delivery.

2.b.1. Polyesters-based nanocarriers

Polyesters such as poly(lactide-co-glycolyde) (PLGA), poly(lactide) (PLA) and poly(ε-caprolactone) (PCL), are the most commonly used polymers for pharmaceutical applications, with PLGA as principal polymer for nanoparticles production [169]. In addition, following our discovery on the positive role of the PLGA PEGylation in protein formulation, a number of studies have adopted this strategy [170,171].

PLGA is a synthetic co-polymer composed of a mixture of two structural monomer units: lactic acid and glycolic acid (the monomers which form respectively PLA and PGA). For the purpose of peptide/protein delivery using PLGA nanoparticles, the cargo can be localized either inside the polymer matrix or attached on its surface (adsorbed or covalently linked) [172,173]. The main interest of these polymers relies on the fact that they are part of a number of marketed formulations, some of them containing peptides [174].

2.b.1.a. Preparation techniques

In general the principles for the formation of these nanoparticles involve the dissolution of the protein in an aqueous phase and the dissolution of the polymer in an organic solvent. The main difference among techniques resides in the nature of the organic solvent in which the polymer is dissolved, and in the composition of the external aqueous phase. In the case that the polymer is dissolved in a non-polar solvent, i.e. ethyl acetate, the protein solution forms an emulsion and this emulsion could be subsequently emulsified in a water phase (double emulsion-solvent evaporation) or precipitated in a polar solvent external phase (emulsion-solvent diffusion). When the polymer is dissolved in a polar solvent the protein is co-dissolved in this phase, and this polar phase can be precipitated upon solvent diffusion in water (nanoprecipitation).On the other hand, a critical step in these fabrication methodologies is the mixing of the different phases. This can be

achieved using minor energy sources (regular agitation) in the case of emulsion-solvent diffusion and nanoprecipitation or high energy sources in the case of double emulsion-solvent evaporation.

Finally, it is important to highlight that the microfluidics approach is currently receiving a great attention as a way to mix the phases. In fact, microfluidic devices can control the way the different phases are mixed with each other, with the possibility of tuning the physicochemical properties of the particles formed [175–177].

Table 7 gives an overview of the techniques employed to produce polyester-based nanoparticles for protein/peptide delivery.

Table 7. Main characteristics of the most commonly used techniques to form peptide/protein-loaded polyester-based nanoparticles

Technique	Principle	Stress exposure	Organic solvents	Simplicity
Double emulsion - solvent evaporation	Double emulsion and precipitation of the polymer due to evaporation of the solvent	Homogenization/ sonication/ surfactants	Yes	+
Emulsion-solvent diffusion	Emulsification and precipitation of the polymer due to the diffusion of the solvent into a non-solvent external phase	Vortex/surfactants (if necessary)	Yes	+
Nanoprecipitation	Polymer precipitation into a non-solvent external phase	No	Yes	+

i) Double emulsion-solvent evaporation

This technique was described in the early 90's for the microencapsulation of proteins, and, a few years later, our group pioneered its adaptation to the encapsulation of proteins within nanoparticles of around 200 nm [178]. The principle involves the emulsification of an aqueous solution containing the protein/peptide into an organic non-polar solvent (i.e. methylene chloride or ethyl acetate) containing the polymer (i.e. PLGA), thereby forming a W/O emulsion. This W/O emulsion is then emulsified again in a volume of an external aqueous phase containing a surfactant (i.e. polyvinylalcohol (PVA)). The solvent present in the resulting double emulsion is eliminated by evaporation [173,178,179]. The two emulsification processes require the use of high energy sources (homogenization, sonication or high speed vortex in the case of small volumes). A schematic view of the procedure is shown in *Figure 16.*

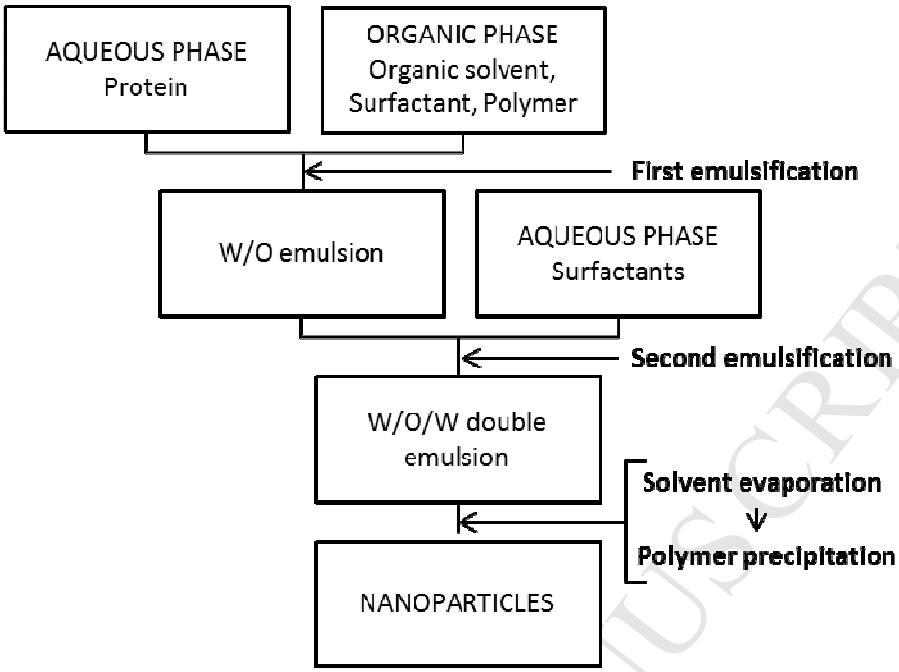


Figure 16. Schematic view of the double emulsion-solvent evaporation procedure to produce polyester-based nanoparticles

ii) Emulsion -solvent diffusion

As in the previous method, an aqueous solution of the peptide/protein is emulsified in an organic non-polar phase containing the polymer and potentially some surfactants. Then, this emulsion is added to an external polar phase (a mixture of water and ethanol) in which the organic solvent is miscible. As a consequence, the polymer precipitates into the polar phase (polymer non-solvents), causing the formation of the nanoparticles (*Fig.17*) [180–183]. A final evaporation step is necessary to remove the organic solvents.

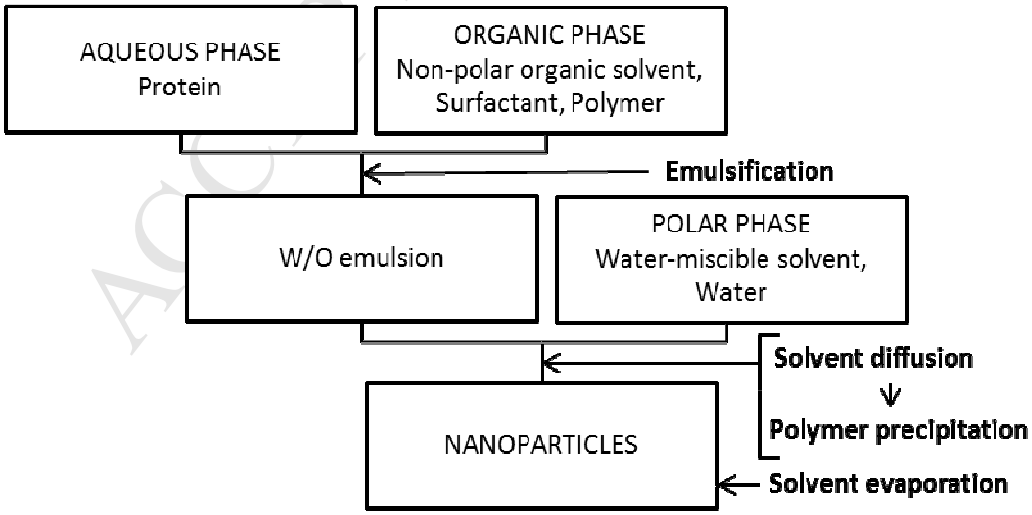


Figure 17. Schematic view of the emulsion-solvent diffusion method to produce polyester-based nanoparticles

iii) Nanoprecipitation

According to this method, the polymer and the proteins are dissolved into a water-miscible organic solvent that is then added dropwise or injected into a dispersing phase in which the polymer is not soluble (*Fig. 18*). The rapid shifting of the solvent into the water causes the nucleation of the polymer, which aggregates, forming the nanoparticles [184,185]. A final evaporation step to remove solvent traces is usually done.

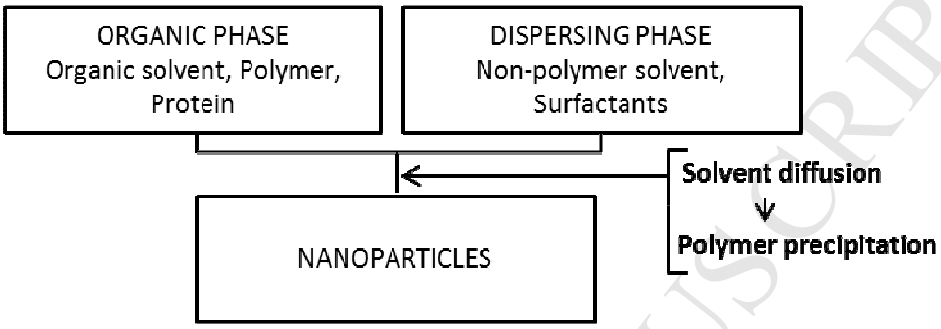


Figure 18. Schematic view of the nanoprecipitation method to produce polyester-based nanoparticles

2.b.1.b. Characterization, peptide/protein loading, activity and release profile

- Particle size distribution: the nanoparticles production technique and the associated formulation parameters have been reported to influence the final particle size distribution. For example, in the case of the double emulsion-solvent evaporation, the size of the particles is highly dependent on the type of instrument and energy applied during the mixing of the organic and aqueous phases. Additionally, the polymer concentration and the type and amount of surfactants added to the formulation may affect the particle size distribution [186,187]. In the case of the solvent-diffusion/nanoprecipitation based techniques, the particle size is mainly determined by the polymer concentration and the rate of mixing the two phases. In general, for protein delivery purposes, particles sizes between 100 and 300 nm and negative surface charges are reported [181,183,185,188].

- Peptide/protein loading and activity: most of the articles reporting the encapsulation of peptides/proteins within PLGA nanoparticles, refer to high AE values, however the final LC is not normally reported and it is usually lower than 5 % [181,182,188]. Both, the AE and LC depend on the preparation technique and also on a number of formulation factors, which include the type of PLGA (ratio lactic/glycolic acid), its molecular weight, its concentration in the polymer solution, the presence of stabilizers or other formulation additives, as well as the type and theoretical loading of the protein. For example, the molecular weight of the polymer and its hydrophobicity have influenced the L-asparaginase loading capacity of PLGA nanoparticles, showing values ranged from 1.8 up to 4.9 % LC [189].The highest LC was achieved with high molecular weight-hydrophilic polymers, which was rationalized as follows. While the presence of free carboxylic groups in the chains of the hydrophilic polymers facilitated its interaction with the protein, the high molecular weights led to a highly viscous polymer solution, which made difficult the diffusion of the protein

from the organic phase to the external aqueous medium. In a different study it was found that the presence of mannosamine covalently attached to PLGA nanoparticles produced by double emulsion/solvent evaporation led to an increase in the association of insulin compared to the unmodified PLGA particles (68 vs 77 % AE; 3.5 vs 4 % LC), probably due to an interaction between the mannosamine residues and the protein [190]. On the other hand, the pH of the internal protein-containing aqueous phase has also been shown to influence the association of BSA to PLGA nanoparticles. Indeed, in a particular study, it was shown that a pH value near the BSA isoelectric point led to a significant increase in the BSA association due to an increase in its hydrophobicity [188].

Finally, it is important to highlight that the presence of stabilizers such as sodium bicarbonate, trehalose or poloxamer 188 in the inner aqueous phase were found to help the stability of the protein during the nanoparticles preparation procedure, although this was normally associated to a decrease in AE values [178,191]. In another case, it was shown that the presence of both, heparin and BSA, as formulation additives was fundamental to increase the association of PDGF-BB (platelet-derived growth factor) (from 35 % to 87 %) into PLGA nanoparticles produced by the solvent diffusion technique. This was attributed to the surfactant properties of BSA, which led to a reduction of the contact of the growth factor with the water/oil interface, thus increasing the association of the protein to the nanoparticles [182].

In fact, the main source of peptide/protein instability common to all the above described techniques is the presence of organic solvents, which can cause denaturation and/or aggregation. The use of stabilizing additives (e.g. methyl-β-cyclodextrins, BSA or PEG) could increase the stability of the drugs, helping them to keep their structure [182,184,185]. Sonication (for double emulsion-solvent evaporation) and the presence of surfactants (for both double emulsion-solvent evaporation and emulsion-solvent diffusion) can also affect the peptide protein/structure [184]. HPLC, ELISA and enzymatic assays have been used to evaluate both encapsulation and structural stability of peptides/proteins [182,184,191]. Direct *in vivo* evaluation of the formulation has also been reported with the same aim [191].

- Peptide/protein release: the typical protein release profile from PLGA nanoparticles consists of an initial burst followed by a sustained release that may last from days to weeks depending on the characteristics of the PLGA nanoparticles. In general, the first fraction of protein released is the one located close to the surface of the particles [181]. Then, the release of the entrapped protein is triggered by the degradation of the polymer by erosion, followed by the diffusion of the protein through the channels created in the process [192]. This erosion process is known to generate oligomers that can easily interact with the encapsulated protein leading to its denaturation [193]. Based on this finding, we have developed a variety of strategies to prevent this critical problem. These include the incorporation of surface active materials, i.e. block copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) [178,181], as well as the use of PEGylated PLGA [170,171]. In both situations, the presence of PEG molecules inside the PLGA matrix was found to work as a barrier for the irreversible deleterious protein-polymer interaction. Finally, although the

mechanism of release is mainly driven by the degradation of the polymer, the nature of the protein may also influence its solubility, its interaction with the polymer and its diffusion across the channels generated in the polymer degradation process [171,183,188,190,194].

Table 8 shows examples of proteins associated to PLGA nanoparticles produced by different techniques.

Table 8.Examples of peptide/protein-loaded PLGA nanoparticles obtained through different preparation methods: drug loading and release properties.

Preparation method	Peptide/ Protein	AE (%)	LC (%)	≤1 h burst / cumulative release (time) - pH medium	Ref.
Double emulsion - solvent evaporation	BSA	70 - 80	0.7 - 0.8	n.a. / 80 % (28 d) pH 7.4	[190]
	Insulin	70 - 80	3.5 - 4	n.a. / 20 % (28 d) pH 7.4	
	Cyclosporine A	60 -90	n.a.	15 - 25 % / 70-90% (24 h) pH 7.4	[195]
	BSA	28 - 88	n.a.	n.a. / 40 - 100 % (28 d) pH 7.4	[178]
	HSA	22 - 33	1.3 - 2.6	n.a.	[196]
	Tetanus toxoid	31 - 37	n.a.	n.a. / 7 - 18 % (1 d) pH 7.4 <7 / 4 - 15 % (4 h) pH 1.2*/7.5*	[170, 171]
	L-Asparaginase	15 - 40	1.8 - 4.9	n.a./15 - 95 % (21 d) pH 7.4	[189]
	Insulin	n.a.	n.a.	n.a. / 70 % (40 d) pH n.a.	[197]
	IGF-1	22 - 43	n.a.	n.a. / 78 % (40 d) pH n.a.	
Emulsion - solvent diffusion	BSA	4 - 60	1 - 4 theor.	60 - 80 % / 80 - 90 % (14 d) pH 7.4	[188]
	IgG	n.a.	1 - 4 theor.	5 - 25 % / 10-30 % (14 d) pH 7.4	
	Insulin	20 - 40	0.2 - 0.4	20 % / 80 % (14 h) pH 7.4	[181]
	PDGF-BB	87	0.01	40 % / 80 % (40 d) pH 7.4	[182]
	FGF-2	68	0.01	40 % / 80 % (40 d) pH 7.4	
Nanoprecipitation	Insulin	14 - 23	0.3 - 0.5	n.a.	[184]
	Lysozyme	35 - 91	0.7 - 1.8	n.a.	
	α-chymotrypsin	11 - 71	2 - 5 theor.	n. a.	[185]
	Cyt-c	72	3.6	n. a. / 100 % (120 d) pH 7.3	

AE: association efficiency (100 x associated peptide mass / total peptide mass); BSA: bovine serum albumin; Cyt-c: horse heart cytochrome c; FGF-2: fibroblast growth factor; HAS: human serum albumin; IGF-1: insulin-like growth factor; IgG: immunoglobulin G; LC: loading capacity (100 x peptide mass /total formulation mass); n.a.: not applicable; PDGF-BB: platelet-derived growth factor; Ref.: references; theor.: theoretical; *Enzyme supplemented.

2.b.2. Acrylic polymers-based nanoparticles

Following the pioneering work of P. Speiser and co-workers on the association of antigens (human immunoglobulin G and tetanus toxoid) to polyacrylamide nanoparticles in 1976 [8], different types of acrylic polymers have been used to produce nanoparticles, including polyacrylic acid, polyacrylamides, polymethylmethacrylates and poly(alkylcyanoacrylates) [198]. These synthetic polymers are considered to be biocompatible and, in some cases, biodegradable polymers

[199,200]. Among them, poly(alkylcyanoacrylates) (PACA) are the most commonly used for preparing nanoparticulate systems and, in particular, for the delivery of proteins. Their nitrile and ester groups are electron attractive functional groups and this property makes the vinyl carbon of the monomer really reactive, hence, able to polymerize in the presence of an initiator. Free radical, anionic or zwitterionic polymerization are the main approaches adopted so far for the production of PACA nanoparticles [200–202]. Overall, despite the early development and attention that these particles received in the past, only a few papers describing their use for protein delivery have been found in the literature.

2.b.2.a. Preparation techniques

Apart from the interfacial polymerization method, which has been mainly used for oily core nanocapsules production, and as such, it was described in the previous section (*Section 2.a.4.a.*), two main strategies (summarized in *Table 9*) have been described to synthesize polyacrylate-based nanostructures: the anionic polymerization and the free radical dispersion polymerization techniques. In both cases, the use organic solvents is avoided, being the main source of protein instability its potential reactivity with the monomer.

Table 9. Main characteristics of the most commonly used techniques to form peptide/protein-loaded polyacrylate-based nanoparticles

Technique	Principle	Stress exposure	Organic solvents	Simplicity
Anionic polymerization	Monomers polymerization due to OH^- groups in the medium	Undesirable reactions drug-monomers	No	+
Free radical dispersion polymerization	Monomers polymerization due to the generation of free radicals and crosslinking	Undesirable reactions drug-monomers-crosslinking agent / Free radicals / UV / Heat	No	+

i) Anionic polymerization

In this technique, the acrylic monomers, a stabilizer and an initiator (OH^- in water) are necessary to form the nanoparticles. The monomers, which are poorly soluble in water, are emulsified into an acidic water solution (pH 2 - 4) containing the stabilizer (typically dextran). Once the droplets are formed, the monomer starts to polymerize thanks to the hydroxyl ions (OH^-) present in the water phase (*Fig.19*). The acidic pH slows down the polymerization rate, thereby controlling the process of particles formation [133,201]. Proteins can be attached onto the surface of the particles, or simply incorporated into the reaction mixture during particles formation [203–207].

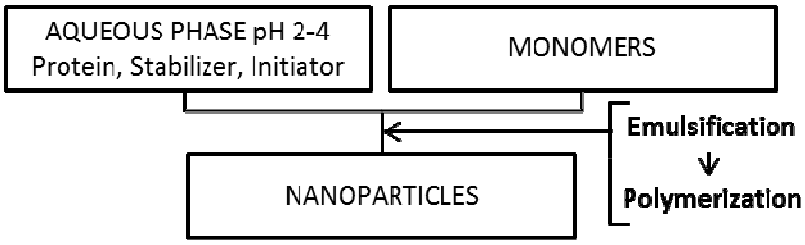


Figure 19. Schematic representation of the anionic-polymerization technique to produce polyacrylate-based nanoparticles

ii) Free radical dispersion polymerization.

Peppas and co-workers used this technique to obtain gel nanospheres through a photo- or thermal-initiated polymerization (*Fig. 20*). This technology involves the use of specific initiators as well as a crosslinking agent. The monomers (i.e. methacrylic acid, MAA and monomethylether monomethacrylate, PEGMA), the crosslinking agent (i.e. tetra (ethylene glycol) dimethacrylate) and the initiator (i.e., 1-Hydroxylcyclohexyl phenyl ketone) are solubilized in an aqueous phase. Once the initiator is activated (UV, heat), the formation of oligomers and crosslinks starts. Finally, since the polymer is not soluble in water, nuclei of polymerization are created leading to the formation of nanospheres (i.e. P(MAA-g-PEG)). Once the polymerization is completed, nanospheres are purified by repeated washing steps to remove the unreacted monomers and the association of the protein (i.e. insulin, OVA) is carried out in a subsequent incubation step [208,209].

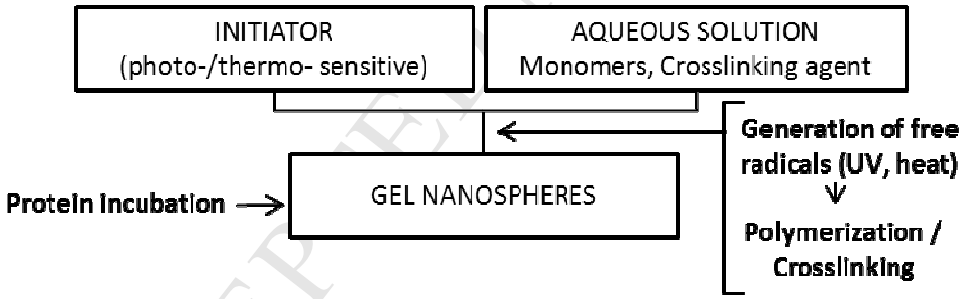


Figure 20. Schematic representation of the free radical dispersion polymerization technique to produce polyacrylate-based gel nanospheres

2.b.2.b. Characterization, peptide/protein loading, activity and release profile

- Particle size distribution: in general, polyacrylate-based nanoparticles described in the literature have a size in the range of 50 nm and 500 nm and a negative surface charge [210–212]. Different parameters can affect the polymerization process and, as a consequence, the physicochemical properties of PACA nanoparticles. The most important parameter, which allows the control of the polymerization rate and, hence the particle formation is the pH, however, the monomer concentration also has a significant influence in this process. Finally, the temperature and the addition of surfactants have also been described as a way to modulate the particle size [203,212–215].

- Peptide/protein loading and activity: *Table 10* gives an overview of the properties of some protein/peptide-loaded polyacrylate-based nanoparticles formulations. The AE and LC values described in the literature are very variable, ranging between 3.5 and 95 % AE and up to 26 % LC [205,206,216]. Among the factors influencing the AE, the time at which the protein is added during the polymerization process has been found to be critical. For example, both insulin and GRF (growth hormone releasing factor) reached around 85 % AE when they were added to the polymerization medium 30 minutes after the process started [135,205].

As for the "*in situ*" polymerization method, the peptide/protein could undesirably work as a monomer during the polymerization procedure, which may result in its inactivation [206,217]. Apart from techniques like HPLC or enzymatic assays [203,217], direct *in vivo* efficacy of the formulation has often been used to test the integrity and activity of the loaded peptides/proteins [209].

- Peptide/protein release: the release of proteins from polyacrylate-based nanoparticles is mainly due to the bioerosion of the polymeric matrix [135]. Typically, these particles show an initial burst release, which can be buffered using additives. The presence of dextran into the formulation medium could, for example, delay the release of BSA from poly(α-butylcyanoacrylate) nanoparticles [206]. Protein release has also been shown to be strongly influenced by the type of PACA used. For example, the release of GRF was faster in the case of poly(isobutylcyanoacrylate), as compared to the case of poly(isohexylcyanoacrylate) nanoparticles. This was due to the different bioerosion rates of the two polymers [135]. In the particular case of the polyacrylate-based gel nanospheres (acrylic acid (AA) or methacrylic acid (MAA), they were specifically designed to exhibit a pH-dependent swelling and, hence, release behavior [209]. This control could be achieved by adjusting the polymerization and crosslinking conditions.

Table 10. Examples of peptide/protein-loaded polyacrylate-based nanoparticles prepared by anionic and free radical dispersion polymerization: drug loading and release properties.

Preparation method	Peptide/ Protein	AE (%)	LC (%)	≤1h burst / cumulative release (time) - pH medium	Ref.
Anionic polymerization	Insulin	87	n.a.	n.a.	[205]
	BSA	3.5	n.a.	15 - 55 % / 70- 90 % (14 d) pH 7.4	[206]
	SOD	7 - 33	n.a.	n.a.	[203]
	NR1	6 - 10	n.a.	n.a.	
	GRF	80	n.a	70 % / 80- 90 % (8 h) pH 7.4*	[135]
Free radical dispersion polymerization	Insulin	65	2.1	n.a.	[208]
		93 - 95	7	10 - 80 % / 100 % (3 h) 1h pH 3 + 2 h pH 7	[209]
	OVA	51	26	0 % / 90 - 100 % (3 h) 1.5 h pH 3 + 2 h pH 7.4	[216]

AE: association efficiency (100 x associated peptide mass / total peptide mass); BSA: bovine serum albumin; GRF: growth hormone releasing factor; LC: loading capacity (100 x peptide mass /total formulation mass); n.a.: not applicable; NR1: anti-glutamate N-methyl D-aspartate receptor 1 antibody; OVA: ovalbumin; Ref.: references; SOD: superoxide dismutase; *Enzyme supplemented.

2.b.3. Polysaccharide-based nanoparticles

The most commonly employed polysaccharides for protein delivery purposes are chitosan, alginate, dextran and hyaluronic acid. Chitosan, a deacetylated form of chitin, is formed by repeated units of D-glucosamine and N-acetylglucosamine [41,42,43]. Alginate is a block co-polymer made by α-guluronic acid (pKa 3.4) and β-D-mannuronic acid (pKa 3.6) residues linearly linked [220]. Like chitosan, it can be chemically modified on the acidic functional groups to obtain the desired properties [221,222]. Dextran is made by α (1→6) glucopyranoside units [223–225]. The hydroxyl groups are the main sites used for chemical modifications, with dextran sulfate as the most common modified form for drug delivery applications [226–228]. Finally, hyaluronic acid is a linear polysaccharide made by repeated units of the disaccharide formed by N-acetyl D–glucosamine and D–glucuronic acid [229]. These natural polysaccharides have in common the property of being water-soluble; however their distinct chemistry results in different pKa and functionality in terms of their potential interaction with different targets and their capacity to be modified with different ligands. Among the polysaccharide-based nanoparticles described so far, those made of chitosan were originally developed in our lab for the association of proteins [15,230]. Since this discovery until now, chitosan nanoparticles have been classified as the polymeric delivery nanoparticles that have received the greatest deal of attention. Overall, an advantage of the techniques for the production of polysaccharide nanoparticles relies in the mildness of the procedures [231–233], with the exception of the chemical crosslinking [234], which may lead to the denaturation of the protein.

Different techniques have been described until now to produce polysaccharide-based nanoparticles and nanocomplexes, being the most commonly employed the ionic gelation and the polyelectrolyte complexation. General specifications of the different preparation techniques are presented in *Table 11*.

Table 11. Characteristics of the most commonly used techniques to form polysaccharide-based nanoparticles containing peptides/proteins

Technique	Principle	Stress exposure	Organic solvents	Simplicity
Ionic gelation/crosslinking	Gelation of the particles by ionic crosslinking	Ionic interactions with the protein / Crosslinking agent	No	+ +
Polyelectrolyte complexation	Ionic interaction between polymers of opposite charge	Ionic interactions with the protein	No	+ +

i) Ionic gelation/Ionic crosslinking

Our lab pioneered the development of chitosan nanoparticles using the ionic gelation/ionic crosslinking technique [15,230], which has been later extended to other polysaccharides such as alginate and dextran [235,236]. This technique is based on the fact that some charged

polysaccharides can gel in aqueous solution in the presence of small ions and crosslinking agents (*Fig.21*) [133,222,237]. The type of gelling agent is different based on the type of polysaccharide. For example, in the case of chitosan, tripolyphosphate (TPP) is the most commonly crosslinking agent employed, while in the case of alginates, the use of calcium salts (calcium chloride, calcium sulfate, or calcium carbonate) is the most common gelation approach [15,222,236,238–241].

Alternatively, nanoparticles can be produced using a chemical cross-linking reaction. However, this technique has not been almost explored for the association of proteins [234] due to the potential chemical reactions with the loaded protein.

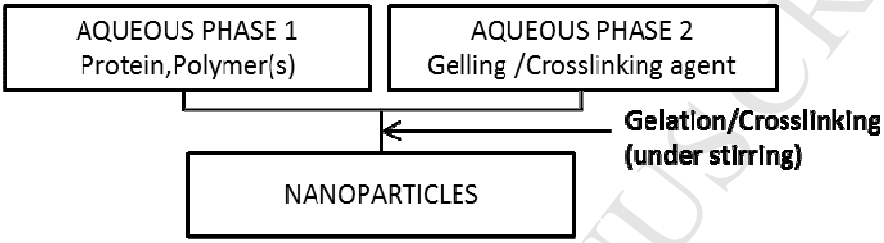


Figure 21. Schematic view of the ionic gelation/crosslinking technique to produce polysaccharide-based nanoparticles

ii) Polyelectrolyte complexation

Polyelectrolytes complexes (PECs) are complexes resulting from the mixing of two oppositely charged macromolecules (i.e., polyelectrolytes). A schematic representation of the procedure is shown in *Figure 22* [242,243]. The density of the charges and the charge distribution over the polymeric chains, in addition to the concentration of the two polyelectrolytes are the main parameters influencing the properties of the particles formed. The control of the ionic strength and pH of the reaction medium, which influences the degree of ionization, is also fundamental for the nanoparticles formation [244].

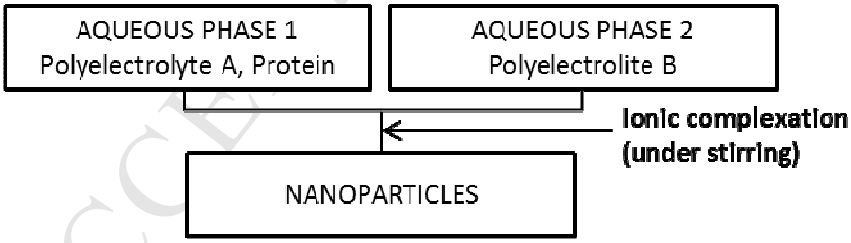


Figure 22. Schematic view of the polyelectrolyte complexation technique to produce polysaccharide-based nanoparticles.

2.b.3.b. Characterization, peptide/protein loading, activity and release profile

- Particle size distribution: the ionic gelation/crosslinking is, among the techniques described above, probably the one allowing a better control of the size. Indeed, in a report by our group [245], intended to compare the ionic crosslinking vs. the ionic complexation of chitosan and pDNA, we showed that the nanoparticles prepared by crosslinking of chitosan with TPP had a more controllable size and a lower polidispersity than those produced by ionic complexation. This result

was attributed to the fact that the crosslinking with TPP led to the formation of nanogelled particles with a round and more defined structure [246,247]. Overall, the main factors influencing the particle size distribution are the ratio and the concentration of the ionically interacting species [15,227].

- Peptide/protein loading and activity: in general, particles produced by gelation or complexation are characterized by a high LC, which can reach values up to 50 % and AE values close to 100 % [15,227,247,248]. The protein association efficiency is mainly affected by the number of interacting species and their degree of ionization. For example the AE of insulin to chitosan nanoparticles reached values close to 90 %, however the value decreased to 37 % in the case of chitosan/glucomannan polyelectrolyte complexes [247]. This was attributed to the different pHs of the protein solution and also to a competition between the protein and glucomannan for the chitosan positive sites. A similar competition phenomenon was observed for the basic peptide salmon calcitonin, which was found to compete with protamine in its association to hyaluronic acid/protamine nanoparticles [249]. These affinity/ionic competition phenomena have been taken into account for the modulation of the LC. For example, the association efficiency of insulin to chitosan-based nanoparticles could be increased from 66 % to 94 % when the anionic interacting polymers were alginate and dextran sulfate respectively. This behaviour was explained due to the strong ionic interactions between the insulin and the sulfate groups of dextran [250].

The main source of instability for the loaded peptide/protein is, in both ionic gelation and polyelectrolyte complexation, the possible ionic interaction between the peptide/protein and the polymers/crosslinking agents, which could drive to protein denaturation [248,251,252]. Additionally, the acidic pH often necessary to produce nanoparticles by ionic gelation (e.g. chitosan nanoparticles) can destabilize or affect the peptide/protein activity (e.g. pH optimum of enzymes) [253]. Both electrophoresis-based techniques (i.e. SDS-PAGE and Western blot) and ELISA assays have been used to check if the peptide/protein integrity and activity were preserved once included in polysaccharide-based nanoparticles[251,252,254]. Likewise, spectroscopy-based techniques like FTIR have been used to study the interactions between the functional groups of the peptide/protein and the polyelectrolytes [255]. In the case of enzymes, the activity was simply evaluated through enzymatic activity assays [256]. Finally, in some cases, the activity was only assessed after their *in vivo* administration [248,257].

- Peptide/protein release: from the point of view of drug release, nanoparticles produced by ionic gelation or complexation normally show an ionic strength-dependent release profile, with an initial burst release. In fact, the sensitivity of these systems to pH changes and to the presence of ions, is one of their main drawbacks [228,247]. An example of this behavior has been observed for insulin-loaded dextran sulfate/polyethylenimine (PEI) nanoparticles produced by complexation, which completely released the peptide in PBS 50 mM after 5 minutes, while just the 65 % of the peptide was released in PBS 5 mM [228].

Among the formulation factors that can be modified in order to have a certain control of the release process, the combination of different counteracting polymers and surfactants can be highlighted. For example, we have shown that the release of BSA from chitosan nanoparticles produced by ionic crosslinking was affected by the presence of poloxamer 188 in the formulation [15,230]. Similarly, Sarmento et al compared the insulin release profile from alginate/chitosan and dextran/chitosan nanoparticles [250]. They showed that the release of insulin was strongly influenced by type of polymers used, being the interaction between the protein drug and the polymers fundamental to control the release. These chitosan/alginate nanoparticles were shown to have a pH-dependent release profile, suitable for the gastric and intestinal environment. In fact, these systems were able to retain the protein at the low pH of the stomach, and release it in the intestine, when the pH increased [236,258]. Swelling, dissociation, diffusion and erosion are reported as the main mechanisms behind protein release from the nanoparticles made by ionic gelation or polyelectrolyte complexation [227,259].

Overall, it could be concluded that polysaccharide-based nanoparticles are those leading to the highest protein loading capacity, among those indicated in this review. The challenge that remains associated to these nanoparticles is related to their limited capacity to control the release in different physiologically relevant media. Nevertheless, the combination of different biomaterials and surfactants are now seen as approaches to overcome this hurdle.

Table 12 reports examples of peptides and proteins encapsulated into polysaccharide-based nanoparticles synthesized by different strategies.

Table 12. Examples of peptide/protein-loaded polysaccharide-based nanoparticles prepared by the different methods: drug loading and release properties

Preparation method	Peptide / Protein	AE (%)	LC (%)	≤ 1 h burst / cumulative release (time) - pH medium	Ref.
Ionic gelation/ crosslinking	Insulin	87 - 97	19 - 55	100 % / 100 % (2 h) pH 4/7 80 - 100 % / 100 % (2 h) pH 6.4	[248]
		40 - 90	20 - 22 theor.	15 - 90 % / 15 - 90 % (2 h) pH 7.4	[247]
	Immuno-modulatory protein P1	10 - 30	16 - 21 theor.	10 -75 % / 10 - 75 % (2 h) pH 7.4	
	BSA	5 - 80	10 - 50	n.a. / 30 - 100 % (8 d) pH 7	[15]
	Tetanus Toxoid	50	10	n.a.	[246]
	VEGF	32 - 94	0.04-0.34	80 % / > 90 % (24 h) pH 7	[254]
	PDGF	27 - 54	0.05 -0.1	n.a. / > 90 % (7 d) pH 7	
	Insulin	69	10	95 % / 95 % (2 h) pH 1.2 80 % / 80 % (2 h) pH 6.8	[250]
Polyelectrolyte complexation	BSA	70	n.a.	40 - 60 % / 40 - 60 % (7 h) pH 7.4	[255]
	Insulin	66 - 94	5 - 13	55 - 100 % / 55 - 100 % (2 h) pH 1.2 70 - 100 % / 70 - 100 % (2 h) pH 6.8	[250]

	rHBsAg	90 - 95	2.5 - 5	n.a.	[252]
	sCT	100	10 - 39	55 % / 70 - 80 % (24 h) pH 7.4	[249]
	TRIAL	n.a.	n.a.	n.a.	[257]
	ARH peptide	36 - 72	11 - 13	n.a. / 15 - 60 % (6 d) pH 7.4	[260]
	OVA	80 - 85	7 - 38	n.a.	[220]

AE: association efficiency (100 x associated peptide mass / total peptide mass); BSA: bovine serum albumin; INF-α: interferon alpha; LC: loading capacity (100 x peptide mass /total formulation mass); n.a.: not applicable; OVA: Ovalbumin; PDGF: platelet-derived growth factor; Ref.: references; rHBsAg: recombinant hepatitis B surface antigen; sCT: salmon calcitonin; theor.: theoretical; TRIAL: tumor necrosis factor-related apoptosis inducing ligand; VEGF: Vascular endothelial growth factor.

2.b.4. Protein-based nanoparticles

Protein nanoparticles have been proposed for a long time as drug delivery systems due to their low cost, easy production, low cytotoxicity and biodegradability [261,262]. A protein nanoparticle-based product for the delivery of paclitaxel (Abraxane®) has been approved by FDA and EMA, generating a high interest around this kind of particles. Recent works related to protein nanoparticles for protein delivery have been reported in literature, using gelatin, HSA, BSA, green fluorescent protein (GFP) and silk fibroin as starting materials to produce the particles [261].

2.b.3.a. Preparation techniques

The preparation method most commonly used to produce protein nanoparticles is the desolvation technique, described below.

i) Desolvation

An aqueous solution of both the therapeutic protein and the one used as a starting material to produce the particles is prepared. A desolvating agent, like acetone, ethanol or dimethyl sulfoxide (DMSO), is then slowly added to the proteins solution. After the desolvation process, nanoaggregates of the proteins are formed and a crosslinking agent, usually glutaraldehyde, is added, causing the formation of stable particles (*Fig.23*) [262,263]. Alternatively to the chemical crosslinking, a coating with an ionic polymer (e.g., PEI) can be done to improve the stability of the particles [264].

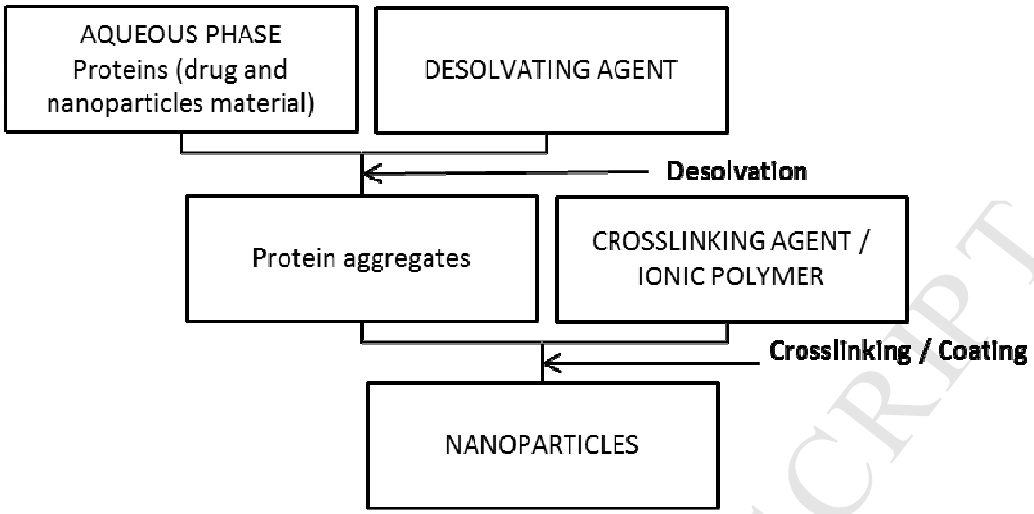


Figure 23. Schematic view of the desolvation technique to produce protein nanoparticles

2.b.3.b. Characterization, peptide/protein loading, activity, and release profile

- **Particle size distribution:** the size of the protein-based nanoparticles, which usually ranges between 150 and 400 nm, depends on parameters like the type of crosslinker and the crosslinking time. Their surface charge depends on the pH of the media and the type of protein used to produce the particles [263–266].

- **Peptide/protein loading and activity:** although the number of references describing the use of protein nanoparticles for protein delivery is very low, in general high AE values are reported in literature (*Table 13*). Furthermore, the presence of a polymer coating that helps to retain the protein drug can also enhance the AE values of protein nanoparticles, as demonstrated for albumin nanoparticles prepared by desolvation with PEI forming the polymer coating [264].

The main drawback of the desolvation process is the use of organic solvents or crosslinking agents, which could denaturate the peptide/protein structure, leading to protein inactivation. In this regard, ELISA and enzymatic assays have been used to check if the peptide/protein activity was retained after the nanoparticle formation [262,264,265].

- **Peptide/protein release:** a first burst release followed by a sustained release profile is usually observed. The sustained release phase is associated to the degradation and dissolution of the protein matrix. Therefore, the release is highly dependent on the type of protein forming the matrix and also on its interaction with the protein cargo [264]. In the case of the PEI-coated BSA nanoparticles developed by Zhang and co-workers, it was observed that a the layer of PEI could reduce the undesired release of the protein drug (bone morphogenetic protein-2, BMP-2) from 70 % to 15 % in the first hour [264].

Table 13. Examples of peptide/protein-loaded protein-based nanoparticles: drug loading and release properties

Preparation method	Peptide / Protein	AE (%)	LC (%)	≤ 1 h burst / cumulative release (time) - pH medium	Ref.
Desolvation	BSA	0 - 89	n.a.	10 % / 90 % (150 h) pH 7.4	[253]
	β-galactosidase	80 - 95	n.a.	25 - 35 % / 40 - 60 % (300 h) pH 7.4	[262]
	BMP-2	> 90	n.a.	10 - 70 % / 50- 80 % (250 h) pH 7	[264]
	VEGF	100	n.a.	5 - 85 % / 50 - 100 % (20 d) pH 7.4	[265]
	HSA	80	10	n. a. / 25 % (400 h) pH 7.4	[266]

AE: association efficiency (100 x associated peptide mass / total peptide mass); BMP - 2: bone morphogenetic protein - 2 BSA: bovine serum albumin; HSA: human serum albumin; LC: loading capacity (100 x peptide mass /total formulation mass); n.a.: not applicable; Ref.: references; VEGF: Vascular endothelial growth factor.

3. Current status of peptide/protein-loaded nanotechnologies

The market of proteins and peptide drugs is growing exponentially, being proteins some of the top selling drugs in the last years, particularly antibodies [267,268]. Despite their potential as therapeutics, the feasibility of using protein drugs to treat patients is often hampered by their short action, inadequate biodistribution and, in general, by the necessity of being administered by injection [267]. As described in this review, numerous attempts have been made in order to overcome these draw-backs through the use of drug delivery nanocarriers [269]. All these efforts have been so far translated into the development of a few protein/peptide-based nanomedicines that are now on the market or under clinical development [20]. Although several strategies have been oriented towards making feasible the administration of proteins following a variety of modalities of administration (ocular, pulmonary, nasal, transdermal) [267,270], the most advanced developments are intended for oral administration and local delivery to the intestinal cavity [271,272], or for systemic delivery upon oral [1,273] or parenteral administration[274].

With regard to the **parenteral modality of administration**, the use of nanodelivery carriers has been found to improve the biodistribution and half-life of proteins (e.g. growth factors, vaccines), thereby enhancing and prolonging their efficacy [274]. Among the nanocarriers investigated, liposomes are the ones that have made their way to the market, in particular in the area of vaccination (*Table 14*). For example, Inflexal® V is a 150 nm liposome formulation that contains influenza virus antigens and is in the market since 1997 [275]. Another liposomal marketed formulation is Mepact®, which contains the immune stimulant polypeptide drug mifamurtide, and was commercialized in Europe in 2009 for the treatment of non-metastasizing resectable osteosarcoma [276].

The possibility of administering peptide drugs by the oral route has attracted a great deal of attention. Nevertheless, the aggressive environment of the gastrointestinal tract and the low permeability of the intestinal epithelium make the administration of peptides/proteins through this route a great challenge [277]. So far there are only two marketed oral peptide formulations intended to achieve a systemic effect. These are Neoral®, a microemulsion formulation containing the hydrophobic peptide cyclosporine A [278], and DDAVP®, approved by FDA in 1992, which is a

simple tablet formulation of desmopressin that has a very limited but sufficient bioavailability (0.1 %) [279]. The efforts devoted in nanomedicine to facilitate the systemic delivery of peptide drugs have been translated into a few formulations, which are now in clinical trials. Two of these prototypes, which are made of inorganic particles, are in an early phase clinical development. However, there is a liposomal formulation intended to deliver insulin to hepatocytes that is currently in phase III clinical trials (Diasome Pharmaceuticals Inc.) [280]. In this case, the liposomes contain a hepatocyte targeting agent (biotin-phosphatidylethanolamine) that facilitates their uptake by hepatocytes upon their absorption through the hepatic-portal vein. It is also worth noting that a number of companies are currently working in advanced preclinical phases on nanoparticulate formulations for oral insulin delivery [281].

Table 14. Some selected examples of peptide/protein-loaded nanoformulations currently in the market or in clinical trials for parenteral or oral administration.

Type of system	Active drug	Indication (route)	Commercial name	Status	Ref.
Liposomes	Influenza virus antigens	Influenza vaccine (SC, IM)	Inflexal® V	Switzerland 1997	[275]
	Mifamurtide	Non-metastasizing resectable osteosarcoma (IV)	Mepact™	EMA 2009	[276]
	HPV E6 + E7 Peptides	HPV-Related Cancers (SC)	n.a.	Phase I	[21]
	Insulin (targeting to hepatocytes)	Diabetes (Oral)	n.a.	Phase III	[280]
Microemulsion	Insulin	Diabetes (Oral)	n.a.	Phase I	[282]
Nanoemulsion (SEDDS)	Cyclosporine	Prophylaxis of organ rejection following organ transplant (Oral)	Neoral®	FDA 1995	[278]

HPV: human papilloma virus; IM: intramuscular; IV: intravenous; n.a.: not applicable; Ref.: references; SEDDS: self-emulsifying drug delivery system; SC: subcutaneous.

Other modalities of peptide/protein administration have also been explored so far with more limited success. For example, the **buccal administration** has been explored for administering peptides such as insulin. In particular, insulin-loaded PEG-b-PLA nanoparticles embedded into a chitosan film were shown to increase the insulin permeation compared to pure insulin *ex vivo* administered [283,284].

On the other hand, the **pulmonary modality of administration** has attracted particular attention due to the huge surface area and high vascularization of the pulmonary mucosa as well as to the highly permeable blood–alveolar barrier. Because of this, two insulin formulations have already been commercialized, one of them withdrawn from the market in a short time [285]. In view of this, some authors have considered that the encapsulation of peptides within nanocarriers may

help to overcome the limitations of the simple powders or solutions that reached the market [270]. Nanocarriers such as liposomes and chitosan-based nanoparticles have been investigated for pulmonary delivery of different drugs, such as insulin, calcitonin, leuprolide, enzymes, cytokines and cyclosporine A [70,286–290]. For example, Al-Qadi et al. demonstrated a pronounced hypoglycemic effect in normal rats after intratracheal administration of a powder consisting of chitosan nanoparticles encapsulated in mannitol [286]. Similarly, Trapani et al., developed heparin-loaded chitosan-based nanoparticles able to deliver the peptide to the lungs *in vivo* to treat thromboembolic disorders [291]. Interestingly, phase I clinical studies have been reached with a liposome-based formulation delivering interleukin-2 as therapeutic protein to treat pulmonary metastases. The formulation was not toxic and showed a good pulmonary delivery after inhalation, although further studies to test the efficacy of the therapy are not yet carried out [289].

Our group has devoted significant efforts to the **nasal administration** of both peptides (i.e. insulin, calcitonin) [119,248,292] and protein antigens [170,293]. Compared to other types of epithelia, the nasal epithelium is rather porous and allows the transport of relatively large molecules However, the nasal delivery of peptides/proteins is hampered by the efficient mucociliary clearance existing in the nasal cavity, which also results in a high physiological variability [294]. We pioneered the development of chitosan nanoparticles specifically designed for nasal insulin delivery [248]. We have also investigated the potential of an array of nanocarriers made of PLGA-PEG and also of chitosan for the delivery of antigens, i.e. tetanus toxoid and hepatitis B [153,170,293,294]. Finally, in this area it should be highlighted the development of a cyclosporine A liposomal formulation whose phase I clinical trials were completed in 2015. The nasal administration of this formulation notably improved the pharmacokinetics of the peptide without showing any side effect [295,296]

Other modalities of administration, i.e. the **ocular and dermal routes** have been explored as potential ways to deliver peptide/protein drugs with the help of nanotechnology. For example, cationic liposomes containing super oxide dismutase were used to treat UV-induced skin damages *in vivo*, showing an enhanced transport of the protein through the skin when liposomes were coupled with iontophoresis [297]. On the other hand, antibodies, growth factors, cyclosporine A and antibiotics are just some examples of proteins and peptides delivered to the eye, following different modalities of administration. For example, a cyclosporine A topical microemulsion formulation was marketed in 2003 with interesting outcomes for the treatment of dry eye [298]. Examples of preliminary preclinical developments include bevacizumab-loaded liposomes associated to the protein annexin A5 [299] and bevacizumab-loaded chitosan nanoparticles, which showed an enhanced anti-angiogenic effect after they were intravitreally injected in rats [300].

4. Conclusions

In this review we disclose a number of technologies and biomaterials that can be potentially used for the delivery of proteins. All these technologies and related biomaterials have specific advantages and disadvantages. From the technological point of view, the use of solvent-free and energy-free approaches, which do not require chemical reactions, are obviously desirable. Lipid microemulsions, nanocapsules and polysaccharide-based nanoparticles are those nanosystems that can be produced according to the mild indicated techniques. However, a limitation of the lipid-based nanosystems is their limited loading capacity, whereas that of polysaccharide-based nanoparticles relies on their limited stability and controlled release capacity. Overall, the conclusion is that despite the important advances in the field, there is still a need to optimize the nanocarriers' properties for the desired peptidic drug through a rational design. In this regard, the capability of the nanocarrier to efficiently entrap, appropriately release and preserve the integrity of the protein/peptide loaded are so far the critical parameters to consider. Up until now, most nanocarriers have been limited in their composition and architectural design. The current design may need to imply the use of a combination of biomaterials conveniently organized within the nanosystems internal and superficial structure. Hopefully, the use of these advanced nanocarriers as delivery platforms will allow in the early future the successful administration of a wide variety of potential therapeutic proteins/peptides.

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