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by enabling to overcome the extracellular and intracellular physiological barriers affecting the delivery. For that, rational formulations and rational process designs are needed. This chapter addresses a comprehensive description and critical appraisal of the main production methods of this particular type of lipid nanoparticles and the leading strategies to prompt a targeted delivery of siRNA.

Keywords (separated by '-') RNA interference - Solid lipid-based nanoparticles - Targeting - siRNA delivery - Temperature-based methods - Solvent-based methods

Chapter 14

Targeted siRNA Delivery Using Lipid Nanoparticles

Andreia Jorge, Alberto Pais, and Carla Vitorino

Abstract

Efficient intracellular delivery of small-interfering ribonucleic acid (siRNA) to the target organ or tissues in the body is assumed as the main hurdle for a widespread use of siRNAs in the clinics. Solid lipid-based nanoparticles (SLNs) and derivatives can potentially fit this purpose by enabling to overcome the extracellular and intracellular physiological barriers affecting the delivery. For that, rational formulations and rational process designs are needed. This chapter addresses a comprehensive description and critical appraisal of the main production methods of this particular type of lipid nanoparticles and the leading strategies to prompt a targeted delivery of siRNA.

Key words RNA interference, Solid lipid-based nanoparticles, Targeting, siRNA delivery, Temperature-based methods, Solvent-based methods

1 Introduction

In the 1990s, Fire and Mello discovered the ability of the small-interfering ribonucleic acid (siRNA) to robustly inhibit the expression of specific genes in *Caenorhabditis elegans* and plants [1]. Since then, RNA interference (RNAi) received a great deal of attention for their application as next-generation medicines with potential to prevent and treat genetic disorders, providing an alternative treatment when conventional drugs fail.

The RNAi mechanism involves the pairing of double-stranded siRNA with a 21-nucleotide (nt) endogenous mRNA. Briefly, siRNA loads in a double-stranded fashion into a gene regulatory complex, known as RNA-Induced Silencing Complex (RISC), which includes three proteins, DICER, Argonaute2, and transactivation response RNA-binding protein (TRBP) [2, 3]. One strand, the sense strand, is discarded, degraded, and released, while the antisense strand is paired to a complementary mRNA through RISC complex. The bound mRNA is then cleaved at a position 10 and 11 nt from the 5′-end of the antisense siRNA by the

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Argonaute2 that is considered the catalytic processor of the RNAi machinery [4, 5].

From a therapeutic perspective, siRNA can be synthetically designed to induce sequence-specific endonucleolytic cleavage of a disease-causing mRNA. Although this approach is widely used in preclinical models, the clinical translation of RNAi is still challenging, because of the difficulty in achieving good biodistribution and pharmacokinetics. Similarly to other oligonucleotides, siRNA faces multiple obstacles before reaching their intracellular site of action, including plasma membrane and intracellular trafficking. Additionally, naked siRNA is relatively unstable in the blood circulation, due to the nonspecific uptake by the reticuloendothelial system (RES) and aggregation with serum proteins, which leads to a rapid clearance from the body by rapid renal excretion following degradation by nucleases. Thus, when designing an effective siRNA delivery strategy, the following requirements must be considered: (1) protection from the enzymatic digestion, (2) enhancement of the pharmacokinetics by avoiding RES uptake and rapid renal filtration, (3) improvement of the translocation through the endothelium, (4) enhancement of the diffusion through the extracellular matrix, (5) improvement of the cellular uptake, (6) intracellular endolysosomal escape, and (7) minimization of potential siRNA-induced toxicity [6–9].

Progresses have been made towards the goal of siRNA application as therapeutic oligonucleotides, recently recognized by US Food and Drug Administration (FDA) with the approval of the first drug based on RNA interference, ONPATTRO™ (patisiran) from Alnylam Pharmaceutics. In addition, at least six other RNAi therapeutics are currently in the late stage of clinical trials (Phase III) [10]. Likewise, European Medicines Agency has also recently approved patisiran. The success of converting siRNA molecules into efficient drugs stems from the development of oligonucleotide chemistries that evolved to increase the resistance to nucleases, increase silencing potency, reduce off-target activity, and avoid innate immune responses [11–13]. However, and more importantly, the incorporation of siRNA molecules into "smart" vehicles that can efficiently escort them into the target cells is a requisite. In this regard, solid lipid-based nanoparticles (including solid lipid nanoparticles and nanostructured lipid carriers) represent a promising candidate for gene delivery.

The matrix of these carrier systems consists of a relatively firm core of physiological lipids stabilized by an aqueous solution of surfactant(s); therefore, to clearly differentiate these particles from other lipid nanostructures, for example, nanoemulsions and liposomes, they are called as solid lipid nanoparticles (SLN) [14]. SLN is made from solid lipids (i.e., lipids that are solid both at room and body temperature, e.g., fatty acids, glycerides, or waxes), while the nanostructured lipid carriers (NLC), considered a second

generation of lipid nanoparticles, result from a blend of solid and liquid lipids (oils), with the blend being solid at body temperature. The addition of the oil compound precludes the formation of perfect lipid crystals, thus creating more imperfections and providing a lipid matrix with enhanced drug-loading capacity and physical stability [15, 16]. These nanostructures also bear a cationic compound, usually a lipid, that electrostatically interacts with the negative charges of the nucleic acids and forms a complex at the particle surface [17]. Alternatively, neutral lipid–siRNA conjugates have become a subject of considerable interest to improve the safe delivery of oligonucleotides and enhance their pharmacokinetic behavior and transmembrane delivery [8].

SLNs and NLCs claim a number of technical advantages as compared to other nanoparticle systems. These include (1) the use of biocompatible lipids, therefore improving cell tolerance to treatment; (2) high encapsulation; (3) protection capacity for nucleic acid cargoes from biological impacts within the blood circulation and at the target site; (4) control over release, ascribed to their solid nature matrix; (5) appropriate storage stability; (6) efficient scaling for large-scale production with a good cost-effective ratio, together with their (7) feasibility to support sterilization, and lyophilization, as secondary processes [9, 18–20].

In this chapter, design considerations of solid lipid-based nanoparticles will be extensively reviewed, with focus on the production methods and particle/siRNA targeting strategies to encourage the scientific community to explore these valuable carriers. Oligonucleotide and lipid chemistries will be briefly discussed and overviewed to highlight their utility to engineer targeted, safe, and efficient lipid-based nanoparticles for siRNA delivery.

2 Materials

Select the appropriate essential excipients for the nanoparticles preparation (*see* **Note 1**). These include solid lipids (as matrix material in the case of SLN) or both solid and liquid lipids (as matrix material in the case of NLC; *see* **Notes 2** and **3**), emulsifier and water. The term lipid is used here in a broader sense and encompasses the classes referred in Table 1. As emulsifiers, all classes of surfactants/cosurfactants have been employed, but physiologically compatible emulsifiers are preferred as stabilizers [24]. Choose the cationic lipid/surfactant considering the minimum amount required, according to the positive charge density-toxicity profile balance (*see* **Note 4**).

Prepare all solutions using ultrapure water (purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature, unless indicated otherwise (according to the stability). Carefully follow all waste disposal regulations for the disposal of waste materials.

t.1 **Table 1**

AU3 **Excipients typically used for lipid nanoparticles production**

	Neutral lipids					
t.2	**Solid lipids**	**Liquid lipids**	**Cationic lipids/ surfactants**	**Surfactants**	**Stabilizers/stealth compounds[a]**	**Targeting compounds**
t.4	Saturated monoacid triglycerides (e.g., tristearin, tripalmitin, trilaurin, and trimyristin)	Fatty acids (e.g., oleic acid)	Octadecylamine	Polysorbates (Polysorbate 20, 60, 80)	PEG conjugates (e.g., DSPE-PEG, PEGylated c-met siRNA [3])	Peptides (CPP)
t.5	Partial glycerides (e.g., glyceryl monostearate, glyceryl Behenate and glyceryl palmitostearate	Short-chain triglycerides (e.g., caprylic/capric triglycerides)	DOTAP	Phospholipids (soybean lecithin, egg lecithin, phosphatidylcholine)	Hyaluronic acid	Proteins (e.g., KSP [21], survivin [9])
t.6	Fatty acids (e.g., stearic acid, behenic acid, palmitic acid, and decanoic acid)		DC-Chol	Poloxamers (Poloxamer 188, 407)	Chitosan	Antibodies
t.7	Waxes (e.g., cetyl Palmitate		Gemini derivatives [6]	Bile salts (sodium cholate, sodium taurocholate, sodium glycholate)	Polyethylene oxide, poloxamer, and poloxamine	Aptamers
t.8	Steroids (e.g., cholesterol)		Synthetic ionizable lipids (e.g., lipid-based serinol derivatives [22])	Cosurfactants (e.g., butanol)		Small molecules (carbohydrates, folate, vitamins)
t.9			Synthetic ammonium quaternary surfactants (e.g., CTAB [23])			Polymers (e.g., hyaluronic acid)

t.10 *DC-Chol* = 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol, *PEG* = Polyethylene glycol, *DSPE* = 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine, *CPP* = Cell-penetrating peptides, *KSP* = Kinesin spindle protein, *DOTAP* = 1,2-Dioleoyl3-Trimethylammonium-Propane

[a]Stabilizers make nanoparticles stealth and improve the longevity in the blood circulation

t.1 **Table 2**
Equipment required according to the technique employed for the production of the nanoparticles

t.2 High-pressure homogenization technique	Melt dispersion technique	Solvent emulsification-evaporation technique	Hydrophobic ion paring	Double emulsion
1. High-speed stirrer 2. High-pressure homogenizer[a]	1. High-speed stirrer 2. Ultrasonicator	1. High-speed stirrer 2. Ultrasonicator 3. Rotavapor	1. Magnetic/mechanical stirrer 2. Ultrasonicator 3. Rotavapor	1. Magnetic/mechanical stirrer 2. Ultrasonicator 3. Rotavapor

t.3

2.1 Equipment

The specialized equipment necessary for carrying out the techniques described in Subheading 3 is indicated in Table 2.

High-pressure homogenizers function as follows: a pump pushes a liquid (the hot pre-emulsion, or cold pre-suspension, as referred in Subheading 3.1.1) with high pressure (100–2000 bar) through a constricted passageway named the gap region (usually in the range of a few microns). The fluid accelerates on a very short distance to a very high velocity (over 1000 km/h), leaves the gap region, and enters the exit region flowing in the direction of the impact ring. After passing through this region, the fluid (nanoemulsion) exits through the outlet [25].

3 Methods

3.1 Preparation of SLNs

The design of solid lipid-based nanoparticles and derivatives (for the sake of generality, the term SLN will be applied in a broader sense to denote this type of carrier systems) for the delivery of siRNA requires in most cases the use of cationic compounds (e.g., lipids or surfactants) that should be incorporated in their formulation to provide proper surface positive charge necessary for the complexation with siRNA. These complexes could be either entrapped in the core or adsorbed on the nanoparticle surface. In the case of the former, a neutral electrostatic complex (1:1 siRNA: cationic lipid charge ratio) is intended. When siRNA is carried at the particle surface, the optimal ratio of cationic SLNs to siRNA must be obtained for maximizing siRNA complexation. Ideally, the formulation of SLNs should be achieved with the least amount of the cationic lipid, without compromising the properties that make them suitable for the delivery of nucleic acids, that is, a sufficient positive charge along with a reasonable colloidal stability. An excess of these components can result in a higher degree of cytotoxicity. There exists a number of successful methods of preparation of SLNs and derivatives for an effective delivery of nucleic acids into target

cells (*see* Table 3). They can be classified into two distinct categories: temperature- and organic solvent-based methods. The former involves generally the use of high temperatures (above solid lipid melting point), while the latter implies the use of organic solvents. The most relevant ones will be described in detail [26].

3.1.1 Temperature-Based Methods

High-Pressure Homogenization

High-pressure homogenization (HPH) is a technique widely used in several research areas, including the pharmaceutical, for example, in the production of parenteral emulsions [39]. The already established HPH large-scale production lines allow to circumvent the lack of scaling up associated to some nanoparticle production methods. It is also a simple and very cost-effective technique. Additionally, HPH leads to a product relatively homogeneous in size, that is, possessing a higher physical stability in the aqueous dispersion [40].

HPH can be used in two different production techniques: at elevated temperature, hot HPH, or below room temperature, cold HPH, including the steps described in what follows.

Hot High-Pressure Homogenization

1. Heat the lipid to ~5–10 °C above its melting point.
2. Mix water, surfactant(s), cosurfactant(s) (*see* **Note 5**), and the cationic lipid, and heat to the same temperature as the lipids.
3. Add the melted lipid(s) in the hot aqueous phase containing the cationic lipid(s) (*see* **Note 6**) and the surfactant(s) under vigorous stirring with a high-speed stirrer to promote the formation of the pre-emulsion.
4. Homogenize the pre-emulsion in a heated high-pressure homogenizer for several homogenization cycles (*see* **Note 7**) to form a hot o/w nanoemulsion.
5. Cool down the hot o/w nanoemulsion to room temperature, to allow the lipid recrystallization and promote the formation of SLNs.
6. Purify the SLN dispersion through, for example, ultrafiltration-centrifugation or dialysis.

Cold High-Pressure Homogenization

1. Heat the lipid to ~5–10 °C above its melting point.
2. Mix water, surfactant(s), cosurfactant(s), and the cationic lipid, and heat to the same temperature as the lipids.
3. Rapidly cool the melted lipids in liquid nitrogen or dry ice.
4. Grind to obtain lipid microparticles (~50–100 μm).
5. Disperse the milled powder in a cold aqueous surfactant solution to form a pre-suspension.
6. Homogenize the pre-suspension in a high-pressure homogenizer at room temperature or below for several homogenization cycles to obtain the nanosuspension of SLN.

t.1 **Table 3**
Examples of solid lipid-based siRNA delivery systems

t.2 Carrier type	Transfected siRNA	siRNA location	Lipid composition	Method of preparation	Coating/ targeting	Clinical application	References
t.3 Cationic SLNs	c-Met-specific siRNAs	Electrostatically associate	Cholesteryl oleate, glyceryl trioleate, DOPE, Chol, and DC-Chol	Solvent emulsification-evaporation	siRNA-PEG	Glioblastoma	[26]
t.4 Cationic SLNs	Kinesin spindle protein RNA	Encapsulated (majority), and electrostatically associate	DOPC/DOTAP/DOPE-PEG2000 or DOPE/ DOTAP/DOPE-PEG2000	Thin film and hydration method	DOPE-PEG2000	Tumor vasculature	[21]
t.5 Cationic SLNs	siRNA-against P-gp	Encapsulated	Witepsol E85, stearylamine	Modified solvent emulsification-evaporation water-in-oil-in-water (w/o/w) double emulsion technique	Pept-Cys (sulfhydryl group)	Brain diseases	[27]
t.6 SLNs	BACE1 siRNA	Encapsulated	Witepsol E 85	Modified solvent emulsification-evaporation water-in-oil-in-water (w/o/w) double emulsion technique	Chitosan and RVG-9R (facilita a transcitose do siRNA)	Alzheimer's therapy	[28]
t.7 Cationic SLNs loaded with PTX	MCL1-specific siRNA and luciferase-specific siRNA	Electrostatically associate	Retinol, DC-Chol, DPhPE, EDOPC, mPEG-DSPE, and glyceryl trioleate	Solvent emulsification method	–	Cancer	[29]
t.8 LNs (nanosomes)	Silencer cy3-labeled GAPDH siRNA	Encapsulated	Cholesterol and DOTAP	HPH and sonication	–	HCV	[30]

(continued)

t.9 **Table 3**
(continued)

t.10 Carrier type	Transfected siRNA	siRNA location	Lipid composition	Method of preparation	Coating/ targeting	Clinical application	References
t.11 LNs (nanosomes)	siRNA TNF-α	Encapsulated	Cholesterol and DOTAP	HPH	–	Cancer and viral infection	[31]
t.12 Cationic SLN loaded with PTX and QDs	Bcl-2 siRNA	Electrostatically associate	Cholesteryl oleate, glyceryl trioleate, DOPE, cholesterol, DC-chol, DSPE-PEG	Emulsification-solvent evaporation method	DSPE-PEG	Cancer theranostics	[32]
t.13 SLN	TNF-α siRNA	Encapsulated (lipid core)	DOTAP, cholesterol, lecithin, and an acid-sensitive stearoyl PEG (2000) hydrazone conjugate (PHC)	Hydrophobic ion pairing (HIP) technique	PHC	Rheumatoid arthritis	[33]
t.14 SLN	siRNA	Encapsulated (lipid core)	DOTAP, lecithin and DSPE-PEG, 1-butanol	Hydrophobic ion pairing (HIP) technique	DSPE-PEG	Controlled release	[7]
t.15 SLN	TRPV1 targeting siRNA	Encapsulated	Glyceryl behenate and stearic acid	Modified cold HPH technique	–	Pain	[34]
t.16 Cationic SLN	VEGF and GFP siRNA	Electrostatically associate	Cholesteryl oleate, glyceryl trioleate, DOPE, cholesterol, and DC-cholesterol	Modified solvent-emulsification method	siRNA-PEG conjugate	siRNA delivery	[35]
t.17 Cationic SLN	Paclitaxel and BCL2 siRNA	Electrostatically associate	Gelucire 50/13 and DOTAP	Solvent-emulsification method	–	Cervical cancer	[36]

t.18	Cationic NLC	DOX + siRNA targeted to BCL2	Electrostatically associate	Precirol ATO 5, squalene, SPC, and DOTAP	Modified melted ultrasonic dispersion method	DSPE-PEG-COOH and LHRH peptide	Lung cancer	[37]
t.19	NLC loaded with PGE2	MMP3 siRNA, CCL12 siRNA, and HIF1A siRNA	Electrostatically associate	Precirol ATO 5, squalene, 5 SPC, and DOTAP	Modified melted ultrasonic dispersion method	–	Idiopathic pulmonary fibrosis	[38]
t.20	SLN and NLC	Survivin-siRNA	Encapsulated (lipid core)	Trilaurin, trimyristin, tripalmitin, tristearin, cetyl alcohol, oleic acid	Melt dispersion technique and sonication	–	Prostate cancer	[9]

t.21 *DOPE* = 1,2-Dioleosyl-*sn*-Glycero-3-Phosphoethanolamine, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC), 1,2-Dioleoyl3-Trimethylammonium-Propane (DOTAP), 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine-*N*-[Methoxy(Polyethylene glycol)-2000 (DOPE-PEG2000), *DSG-PEG2000* = 1,2-Distearoyl-*sn*-glycerol, methoxypolyethylene Glycol 2000, *PTX* = Paclitaxel, *DPhPE* = 1,2-diphytanoylsn-glycero-3-phosphatidylethanolamine, *DC-Chol* = 3b[*N*-(*N*0,*N*0-dimethylaminoethane)-carbamoyl] cholesterol, *EDOPC* = 1,2-Dioleoyl-*sn*-glycero-3-ethylphosphocholine, and *mPEG-DSPE* = Methoxypolyethylene glycol 2000-distearoyl phosphatidylethanolamine, *HCV* = Hepatitis C virus, *QD* = Quantum dots, *PHC* = Polyethylene glycol (2000)-hydrazone-stearic acid (C18) derivative, *TNF-α* = Tumor necrosis factor alpha, *SPC* = Soybean phosphatidylcholine, *LHRH* = Luteinizing hormone-releasing hormone, *DSPE–PEG–COOH* = 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy (polyethylene glycol)-2000] (ammonium salt), *pept-Cys* = Thr-His-Arg-Pro-Pro-Met-Trp-Ser-Pro-Val-TrpPro-Cys, *RVG-9R* = Short peptide derived from rabies virus glycoprotein

7. Purify the SLN dispersion through, for example, ultrafiltration-centrifugation or dialysis.

Melt Dispersion

This technique is similar to the hot high-pressure homogenization, differing in the homogenizing principles that are underlined. The main steps are described in what follows:

1. Heat the lipid to ~5–10 °C above its melting point.
2. Mix water, surfactant(s), cosurfactant(s), and the cationic lipid, and heat to the same temperature as the lipids.
3. Add the melted lipid(s) in the hot aqueous phase containing the cationic lipid(s) and the surfactant(s) under high-shear homogenization to promote the formation of a hot o/w nanoemulsion.
4. If necessary, sonicate the obtained hot nanoemulsion in order to reduce particle size and narrow the size distribution [23].
5. Cool down the hot o/w nanoemulsion to room temperature, to allow the lipid recrystallization and promote the formation of SLNs.
6. Purify the SLN dispersion through, for example, ultrafiltration-centrifugation or dialysis.

3.1.2 Solvent-Based Methods

The solvent emulsification-evaporation is a method similar to the production of polymeric nanoparticles by solvent evaporation in o/w emulsions, comprising the following steps:

Solvent Emulsification/ Evaporation

1. Dissolve the lipids in an organic solvent immiscible with water (e.g., chloroform or methylene chloride).
2. Prepare an aqueous solution containing the surfactant(s) and the cationic lipid.
3. Disperse (emulsify) the organic solution in the aqueous phase containing the cationic lipid(s) and the surfactant(s) under high-shear homogenization. For guidance, please *see* [41].
4. If necessary, sonicate the obtained emulsion in order to reduce particle size and narrow the size distribution (*see* **Note 8**).
5. Remove the organic solvent using a magnetic stirring or a Rotavapor under reduced pressure in order to promote the lipid precipitation in the aqueous medium and the formation of the SLNs.
6. Purify the SLN dispersion through, for example, ultrafiltration-centrifugation or dialysis.

Hydrophobic Ion Pairing

The hydrophobic ion pairing (HIP) is an approach that enables to overcome the challenge of loading siRNA within SLNs. Accordingly, a drug-surfactant complex is first formed, which provides

lipophilicity enough for incorporation of the siRNA in the lipid core of SLN [7, 42]. This technique comprises the following steps:

1. Dissolve siRNA in RNAse-free water and a cationic lipid (usually DOTAP) in chloroform.
2. Add the DOTAP solution dropwise to the siRNA solution while stirring (*see* **Note 9**).
3. Briefly sonicate in a water bath sonicator (*see* **Note 10**) and mix it with an appropriate volume of methanol to form a single-phase solution.
4. After incubation at room temperature (ca. 1 h), extract the siRNA/DOTAP complexes into chloroform by phase separation.
5. Separately, dissolve the lipid matrix (e.g., lecithin and cholesterol) in chloroform.
6. Add this solution dropwise to the siRNA/DOTAP complexes in chloroform while stirring.
7. If applicable, add PEG derivatives (e.g., polyethylene glycol (2000)-hydrazone-stearic acid (C18) derivative (PHC) and polyethylene glycol (2000)-amide-stearic acid (C18) derivative (PAC) [42], also previously dissolved in chloroform dropwise to the siRNA-lipids mixture.
8. Dry the resulting mixture under nitrogen gas.
9. Dissolve the solid residual in an appropriate volume of organic solvent (e.g., tetrahydrofuran).
10. Add the previous solution dropwise into water while stirring to form nanoprecipitates.
11. Stir the resultant nanoparticle suspension (SLN) at room temperature for a sufficient time to facilitate the evaporation of organic solvent alternatively using a Rotavapor.
12. Purify the SLN dispersion through, for example, ultrafiltration-centrifugation or dialysis.

Double Emulsion

The preparation of SLN through the solvent emulsification evaporation method based on the water-in-oil-in-water (w/o/w) double emulsion technique usually involves the following steps:

1. Dissolve the lipid(s) (*see* **Note 11**) in an appropriate organic solvent immiscible with water (e.g., chloroform, methylene chloride).
2. Dissolve the siRNA in RNAse-free water.
3. Disperse (emulsify) the aqueous phase containing the siRNA in the organic solution including the lipid(s) under high-shear homogenization or sonication to form the primary emulsion (w/o) (*see* **Note 12**).

4. Prepare an aqueous solution containing the surfactant(s) and, if applicable, cationic lipids.
5. Disperse the primary emulsion (w/o) into an appropriate volume of the aqueous surfactant(s) solution under high-shear homogenization or sonication to obtain the double emulsion (w/o/w).
6. Remove the organic solvent using a magnetic stirring or a Rotavapor under reduced pressure in order to promote the lipid precipitation in the aqueous medium and the formation of the SLNs.
7. Purify the SLN dispersion through, for example, ultrafiltration-centrifugation or dialysis.

3.1.3 Methods Overview

A global appraisal of the promising fabrication techniques of the solid lipid-based nanoparticles applied to siRNA delivery is displayed in Table 4. The feasibility of the potential scale-up of these methods to the industrial environment is also addressed.

3.2 Loading siRNA into SLN

siRNA loading into SLN typically involves direct complexation of siRNA molecules to the surface of the preformed cationic carriers. However, after abandoning the carrier protection, siRNA molecules rapidly degrade, and the lack of true encapsulation will likely result in the loss of siRNA in circulation. The same happens once these molecules enter the target cells, resulting in a sharp and rapidly decaying siRNA profile due to lack of control over release. Such a trend will cause short RNAi action, implying a more frequent administration. Considering that siRNA is effective at low level, a substantial fraction of the quickly discharged siRNA will be consequently wasted, and the high levels of intracellular siRNA could be associated to toxicity [9]. Moreover, when siRNA is loaded electrostatically adsorbed at the particle surface, an excess of positive charge (cationic compound) is necessary, which in turn incurs into additional toxicity. The encapsulation of siRNA within SLNs to provide a sustained release of siRNA is thus highly desirable. Notwithstanding, the hydrophobic nature of SLNs impedes efficient loading of hydrophilic drugs, such as oligonucleotides. This issue could be overcome by previously forming electrically neutral siRNA-cationic lipid/surfactant complexes, in order to facilitate their loading into SLNs [7]. A summary of pros and cons and a description of both siRNA-loading strategies into SLNs are presented in Table 5 and in the following sections, respectively.

3.2.1 Coating of SLNs with siRNA via Electrostatic Interactions

1. Prepare siRNA/SLN complexes using a range of molar ratios considering the ratio of amine groups (N) of the cationic lipid to phosphate groups (P) of the siRNA. This charge ratio gives

t.1 **Table 4**

Pros, cons, and process parameters that require optimization to prepare solid lipid-based nanoparticles, according to the technique employed

t.2 Method type	Techniques	Process parameters for optimization	Pros	Cons
Temperature-based methods	High-pressure homogenization	– Homogenization cycles (typically 3–5 passes) or homogenization time, depending to the homogenization type – Homogenization pressure	– Organic solvents avoided – Applied to high lipid content (up to 40%) dispersions – Easily upscalable *Hot HPH technique*: – Smaller particle sizes and narrower size distributions *Cold HPH technique*: – Reduced thermal exposure of the sample	– High energy required *Hot HPH technique*: – Drug and carrier degradation acceleration – Drug partition to the aqueous phase favored *Cold HPH technique*: – Larger particle sizes and broader size distributions
t.3	Melt dispersion	– Time and velocity of homogenization – Time and intensity of ultrasound (if applicable)	– Use of widespread and easy to handle lab equipments	HSH—Dispersion quality Often compromised by the presence of microparticles *US*—Potential metal contamination
t.4				High energy required
Solvent-based methods	Solvent emulsification-evaporation	– Time and velocity of homogenization – Time and intensity of ultrasound (if applicable)	– Thermal stress avoided – Use of widespread and easy to handle lab equipments	– Use of organic solvents – Limited solubility of the lipid in the organic solvent
t.5	Hydrophobic ion pairing	– Time and velocity of homogenization – Time and intensity of ultrasound (if applicable)	– Thermal stress avoided – Use of widespread and easy to handle lab equipments	– Use of organic solvents – Limited solubility of the lipid in the organic solvent
t.6	Double emulsion	– Time and velocity of homogenization – Time and intensity of ultrasound (if applicable)	– Applied to hydrophilic compound encapsulation – Thermal stress avoided	– Use of organic solvents – Higher polydispersity
t.7				

t.8 *HPH* = High-pressure homogenization, *HSH* = High-shear homogenization, *US* = Ultrasonication

t.1 **Table 5**
Advantages and drawbacks of siRNA-loading strategies into SLNs

t.2 siRNA loading into SLN strategy	Advantages	Drawbacks
Electrostatically complexed in the surface	– Simple preparation – More adequate for local delivery	– More susceptible to degradation – Reduced control over release – Possible siRNA dose dumping and potential toxic effects – Toxicity associated to cationic lipid
t.3 Encapsulated	– Higher protection – Sustained release – More adequate for intravenous administration – Reduced toxicity associated to cationic lipid[a]	– Complex formulation – Risk of siRNA degradation during SLN preparation[b]

t.4 t.5 [a]A reduced toxicity associated to the cationic lipid is predicted, since it is entrapped in the core, and duly neutralized with siRNA (1:1 ratio), in contrast to the higher amount required and surface exposition observed when siRNA is complexed at the surface
[b]If proper salt and mild temperature conditions are not provided

an indication of the ionic balance of the complexes and it can be calculated by

$$\text{N/P ratio} = \frac{[\text{Ammonium groups from cationic lipid}]}{[\text{Phosphate groups from siRNA}]}$$

2. For that, add a fixed volume of siRNA aqueous solution at a fixed concentration to a fixed volume of SLN dispersion at variable concentrations, depending on N/P ratio selected. The order of addition should be kept constant throughout experiments (*see* **Notes 13** and **14**).
3. Vortex the final solution and incubate for 30 min at 37 °C to allow siRNA binding to positively charged SLNs.

3.2.2 Encapsulation of siRNA into SLNs

1. Prepare a complex electrically neutral of cationic lipid-siRNA (1:1 charge ratio) (*see* **Note 13**).
2. Add the previous electrically neutral complex to the lipid/organic phase.
3. Disperse into the aqueous phase and homogenize according to the method selected (*see* Subheading 3.1).

3.3 Targeted siRNA-SLN

Despite the substantial advances in siRNA technology, currently available systems still demand more optimization. The key for successful optimization is substantially dependent on developing improved carriers for the efficient and safe siRNA delivery to a target tissue/organ. The current optimization steps focus mainly on improving the stability of siRNA in the circulation, enhancing

tissue targeting and cellular uptake, and improving endosomal escape. As shown in Table 3, PEG conjugation to siRNA or lipids is the most common strategy used to reduce the risk of siRNA degradation that along with the encapsulation or adsorption of this genetic material on SLNs should be able to protect the cargo. Nevertheless, there exist an extended knowledge in the field of oligonucleotide chemical modification that could provide additional protection of siRNA, reduce its immunostimulatory activity, and minimize unwanted off-target effects, and it is important to consider their utility in in vivo applications. Alongside with these chemical modifications, careful design of covalent strategies for linking siRNA and targeting moieties to reach specific sites of intended action in body is also a requisite for achieving successful silencing activity. In this section, a comprehensive enumeration of the strategies developed so far for the enunciated purposes will be presented. These will be directed either to siRNA modification or engineered SLN surface.

3.3.1 *Stability in the Circulation*

PEG Conjugation

PEG increases the colloidal stability and the water solubility of nanoparticles (NPs) by forming a protective hydrophilic layer on the surface of NPs that reduce their aggregation tendency and interaction with blood components. As a result, this phenomenon decreases the opsonization phenomenon and the uptake of NPs by the macrophages of the mononuclear phagocyte system prolonging the blood circulation time [43]. Furthermore, PEG surface modification is helpful to the incorporation of active targeting ligands, which allows for the development of effective antitumor therapeutic strategies [44]. PEG can be conjugated with (1) a lipid, for example, DSPE amine, and in this case, it is added directly to the oily/organic phase [7, 32] or aqueous phase [37] (depending on the technique employed; *see* Subheading 3.1) in the SLN preparation, or (2) directly conjugated with siRNA through a disulfide bond [25]. The latter option comprises two sequential steps: the conjugation of siRNA with PEG and incubation with SLN. The conjugation of siRNA with PEG can be also performed by using a linker (e.g., 3′-hexylamine) to connect the two molecules [26]. For guidance, please *see* refs. 26, 45.

siRNA Chemical Modification

Most siRNAs used currently are chemically modified following phosphoramidite approach as single-stranded RNA and then are hybridized into double-stranded fashion. The incorporation of a variety of natural and artificial modifications into the siRNA strands may allow to solve the problems inherent to in vivo administration, including nuclease degradation, and also enhance siRNA potency and specificity [46–48]. These modifications are typically performed on the internucleotide phosphate linkage through the replacement of the non-bridging oxygen with, for instance, sulfur (PS) (phosphorothioate) [49, 50], boron (boranophosphate) [51],

or methyl (methylphosphonate) groups [52]. In addition, modifications at 2′-position of the ribose can also reduce nuclease degradation while increasing duplex stability and offering protection from immune activation. At this position, the most common modifications are 2′-fluoro (2′-F), 2′-O-(2-methoxyethyl) (2′OMe), 2′-O-(2-methoxyethyl) RNA (MOE), and 2′-fluoro-β-D-arabinonucleotide (FANA) [53–55]. Another modification of interest at this level is siRNA with locked nucleic acids (LNA), which consists of a methylene bridge that connects the 2′-O with the 4′-C of the nucleobase and helps not only to increase the resistance of siRNA to nucleases but also to increase the potency of siRNA [56].

3.3.2 Tissue Targeting and Cellular Uptake

Indeed, targeted delivery of anticancer drugs to cancer cells and tissues is a widely exploited field due to its potential to spare normal/healthy ones. Based on the growing knowledge in cell biology, it is recognized that there are many overexpressed receptors in cells that can mediate the internalization of specific ligands and their cargoes. Taking advantage from cell-type-specific fingerprint, many smart nanoparticles have been designed to incorporate specific moieties that can bind to the receptor docking sites [57–59]. To achieve the desired selectivity, aptamers, antibodies, peptides, proteins, carbohydrates, and small molecules, such as folate and vitamins, are considered suitable candidates to act as recognition modules. The advanced chemistries developed so far for the functionalization of oligonucleotides offer great opportunities to combine these specific modules with siRNA [60]. Procedures for each of these alternatives will be referenced for guidance.

Aptamer chimeras are synthetic single-stranded DNA or RNA molecule with high affinity and specificity to cell receptors or proteins with large application in diagnostic and therapeutic field [61, 62]. Their structure is strategically selected and optimized in vitro by a procedure known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [63]. To date, multiple chemical approaches have been developed for conjugating siRNAs and aptamers [64]. Aptamers can be used in three different ways to deliver siRNA: (1) covalently linked by a small spacer, (2) form a chimera, and (3) electrostatically or covalently combined to nanoparticles. Aptamer-mediated targeted systems have been used to deliver therapeutic oligonucleotides such as siRNA, miRNA, or antisense DNA and proved to be valuable to improve the specificity of nanoparticles. For example, McNamara and colleagues developed an aptamer-siRNA chimeric RNAs for the treatment of prostate cancer in which the aptamer portion of the chimera mediates the binding to a specific cell receptor overexpressed in prostate cancer cells whereas the siRNA modulates the expression of survival genes [65].

Small, larger peptides and protein-based targeting moieties excel in mediating cell-specific delivery of siRNA. Among these

natural macromolecules, cell-penetrating peptides have large applicability due to their ability to cross biological barriers, so as cell membrane and blood-brain barrier [66, 67], and they may be obtained from natural or synthetic sources [68]. Electrostatic complexation of nucleic acids with CPP has demonstrated to assist oligonucleotide nuclear delivery [69]. Covalent strategies were also exploited for conjugating CPPs with siRNA oligonucleotides in an attempt to increase the efficacy of siRNA delivery and reduce the risk of CPP dissociation in physiological fluids. Various types of siRNA-CPPs conjugates were developed using, for example, Penetratin, Tat, Transportan, and melittin peptide, among others [70]. It is established that properties such as the biological activity of siRNA-CPPs conjugates, cellular uptake, intracellular localization, and cytotoxicity are dependent on the kind of CPP used as well as on the length of the cationic peptide [71].

Another important class of molecules able to bind selectively to cell receptors are carbohydrates. These molecules are included in distinct biological processes including cell surface recognition through lectins and specific binding to proteins. Galactose (Gal) has demonstrated to be valuable for the delivery of siRNA to hepatocytes by targeting cell surface lectins, the asialoglycoprotein receptors [72], and have been extendedly exploited [73–75]. In particular, the administration of siRNA-conjugated triantennary *N*-acetylgalactosamine (GalNac) is currently being evaluated in a Phase III clinical trial for the treatment of a rare neurodegenerative disease [76, 77]. Another important carbohydrate derivative used to direct cellular uptake is the hyaluronic acid (HA), a glycosaminoglycan polymer ubiquitously found in extracellular matrix. Chemical modification of HA with functional groups to achieve novel HA derivatives with enhanced properties for drug, gene, and protein delivery has been also exploited [78]. Surface modification of cationic liposomes with biocompatible HA enhances their efficacy by mediating active CD44 targeting in cancer cells while augmenting their circulation time [79]. Hyaluronan-grafted lipid-based nanoparticles have been reported for the delivery of anti-P-glycoprotein (P-gp) and luciferase siRNAs, having shown to target the cancer cells efficiently and specifically reduce mRNA and P-gp protein levels when compared with control particles [80].

3.3.3 *Endosomal Escape*

Once SLN-siRNA complexes internalize cells through endocytosis, they stay trapped in endosomes. Nevertheless, siRNA should reach the cytoplasm to be able to achieve the expected silencing activity. The endosomal uptake pathway is considered to be a rate-limiting barrier in intracellular delivery, especially for oligonucleotides that due to their large size and high negative charge fail to reach the cytoplasm of cells [81]. The accepted mechanisms for promoting endosomal escape are (1) membrane destabilization and pore

formation, which generally occurs with fusogenic peptides and ionizable polycations, (2) rupture, which typically occurs with highly ionizable polycations, and (3) membrane fusion, which commonly occurs with lipid nanoparticles containing fusogenic lipids. Reference [82] provides a compilation of the most relevant methods used to follow the endosomal escape of nanoparticles and a complete description of the associated mechanisms.

Fusogenic peptides, for example, GALA and HA2-penetratin peptides [83, 84], undergo a structural change in response to acidification of the endosome which stimulate to release from endosome. Likewise, the change in the arrangement of pH-sensitive lipids from lamellar to hexagonal phase, as it occur for, for example, DOPE, also causes the destabilization of the endosomal membrane and triggers the release of siRNA to the cytoplasm. Within this class, cholesterol and PEG-lipid conjugates have shown a crucial role in the fusogenicity and pharmacokinetic properties of liposomes [85, 86]. Cationic polymers with ionizable amino groups, such as polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimer, are also powerful candidates to induce the rupture of the endosomal membrane. Many mechanisms have been proposed to explain the effect of cationic polymers with ionizable amino groups on endosomes, but the more accepted hypothesis is the so-called proton sponge that triggers the rupture of endosome through osmotic swelling [87]. PEI is one of the most efficient polycation used for siRNA delivery, due to its great ability to compact RNA combined with its excellent buffering capacity. However, this polycation is highly cytotoxic which hampers its application in in vivo settings [88]. The selection of low molecular weight and branched PEI or the use of PEI polymers composed by low molecular weight oligoamines bound with different reducible cross-linkers may represent a solution to reduce its inherent cytotoxicity [89, 90].

4 Notes

1. The optimization of solid lipid nanoparticles benefits from a relevant design of experiments. This methodology elucidates the effects of many factors (composition and process parameters) simultaneously, also enabling to assess their relative importance and to determine whether the factors interact [91]. When optimizing a formulation or process, there are a number of different methods for tackling the problem, and the resulting data may also be analyzed in a number of different ways. In what concerns experimental designs, a rough classification into screening designs, response surface designs, and mixture designs can be carried out. Screening designs, for

example, fractional factorial and Plackett-Burman designs, allow screening a relatively large number of factors in a relatively small number of experiments. They are used to identify the most influencing factors affecting the system, being applied in the context of optimizing processes. Most often, the factors are evaluated at two levels in these designs. In turn, response surface designs are applied to find the optimal factor settings, while mixture designs are used to optimize, for instance, the excipients composition in formulations [92]. For guidance, please *see* [93].

2. When NLCs are selected for siRNA condensation, the lipid choice is dictated by preliminary studies to assess physical compatibility between solid and liquid lipids. For that, prepare mixtures of the solid and liquid lipid in a ratio of 1:1 in different glass tubes. Melt the lipid mixture, shake, and allow to solidify at room temperature. Analyze visually the glass tubes for the absence of separate layers in the congealed lipid mass. Additionally, smear the congealed mixtures of solid-liquid lipid over a glass slide and examine them microscopically [94].
3. A higher liquid lipid content, in relation to the solid lipid, generally results in an improved delivery achieved by a decrease in particle size (typically below 100 nm). This type of carriers system has been named ultrasmall nanostructured lipid carriers [95].
4. If a cationic surfactant is used, one-tailed cationic surfactants are generally more cytotoxic than the two-tailed surfactants, whereas the amino acid corresponding derivatives and cationic lipids are well tolerated [23].
5. Lipophilic emulsifiers (e.g., soya lecithin) are added to the oily phase, while hydrophilic (o/w) emulsifiers (e.g., polysorbates) are added to the aqueous phase.
6. If a cationic lipid is used, it can be alternatively dissolved in the oily phase rather than in the aqueous phase [23].
7. When high-pressure homogenization is used for SLN production, the number of cycles/time and pressure of homogenization should be optimized according to the formulation composition. Increasing the homogenization period does not necessarily result in particle reduction. Instead, size enlarging due to particle coalescence usually occurs, because of the high kinetic energy of the particles.
8. In the solvent emulsification/evaporation technique, the emulsification step can be supported by ultrasonication, followed by high-shear homogenization or vice versa.
9. Add siRNA and a cationic lipid (e.g., DOTAP) in an appropriate charge ratio (1:1, i.e., one DOTAP molecule per phosphate

group on the siRNA). In this mixture, DOTAP binds to siRNA and forms a hydrophobic ion pair. The siRNA/DOTAP complex is tightly held together by electrostatic interaction between the negatively charged phosphodiester backbone and the positively charged DOTAP headgroup, while the DOTAP hydrophobic domains facilitate efficient encapsulation of the siRNA/DOTAP complex in the lipid nanoparticle [96].

10. The sonication step used to aid the formation of siRNA/DOTAP complex should be carried out using a water bath sonicator and not a tip sonicator and for a short period of time (seconds), so as to prevent possible siRNA degradation.
11. When using the hydrophobic ion pairing (HIP) technique, the incorporation of the siRNA/DOTAP complexes into SLN can be alternatively carried out by another solvent-based method, for example, nanoprecipitation/solvent displacement technique, similar to that employed in the polymeric nanoparticles preparation. For guidance, please refer to [7].
12. The addition of w/o surfactants may be needed to provide stabilization of the primary emulsion.
13. A careful selection of buffer conditions should be carried out considering the role of pH and ionic strength in the electrostatic interaction [97–99]. Rational siRNA design is a required step in order to increase nuclease resistance and reduce off-target effects. Chemical modifications are strategically used to optimize siRNA pharmacokinetic properties and bioavailability (*see* Subheading 3.3.1).
14. Complexes are preferably formed with a slight excess of positive charge to allow them to interact with the negatively charged cell surface. Additionally, size and charge depend on the weight ratio between the particle and siRNA [17].
15. PEG is used to prevent the interaction of drug carrier molecules with insoluble blood proteins that would otherwise accelerate the clearance of nanoparticles. This is a beneficial property of PEG for siRNA delivery provided the role of DOTAP is not overshadowed by the presence of PEG. Thus, DOTAP to PEG ratio should be optimized to achieve desired results [21].

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