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Review

Application of Saccharide Cryoprotectants in the Freezing or Lyophilization Process of Lipid Nanoparticles Encapsulating Gene Drugs for Regenerative Medicine

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Abstract: Lipid nanoparticles (LNPs) have emerged as highly efficient drug delivery systems in gene therapy and regenerative medicine and have demonstrated great potential in recent years. Notably, LNPs encapsulating mRNA vaccines have achieved remarkable success in combating the COVID-19 epidemic. However, LNPs encapsulating mRNA encounter issues of physical and chemical instability and need to be stored and transported under harsh conditions. Lyophilization technology, which is commonly used to increase the stability of nanomedicines, has been increasingly applied to stabilize mRNA-LNPs. Appropriate cryoprotectants, such as saccharides, glycerin, and dimethyl sulfoxide (DMSO), need to be added to mRNA-LNPs during the freezing or lyophilization process to effectively preserve the physical and chemical properties of mRNA-LNPs, ensuring their stability. Saccharides (i.e., sucrose, trehalose, and maltose) are the most widely used cryoprotectants to protect the integrity of mRNA-LNPs. This is because saccharides are relatively safe molecules compared with other chemical molecules for cells and animals. However, different saccharides have varying levels of protective effects on mRNA-LNP formulations, and the optimal saccharide concentration varies depending on the specific mRNA-LNP. This article reviews the application and mechanisms of saccharide-based cryoprotectants in the freezing or lyophilization process of LNP-delivered gene therapies and regenerative medicines, offering guidance for selecting the most appropriate saccharide-based cryoprotectants for mRNA-LNP drugs during freezing or lyophilization processes.

Keywords: cryoprotectants; stability; lipid nanoparticles; drug delivery; regenerative medicine

1. Introduction

Lipid nanoparticles (LNPs) are typically solid or liquid particles composed of lipids, engineered specifically for the encapsulation and delivery of nucleic acids such as mRNA or siRNA [1]. LNPs are generally designed to be more stable in circulation, enable efficient encapsulation of nucleic acids, and enhance cellular uptake [2]. They often contain ionizable cationic lipids, which are crucial for packaging and protecting the nucleic acid cargo.

Liposomes, on the other hand, are vesicular structures made of lipids, typically forming one or more lipid bilayers around an aqueous core. Liposomes are considered one of the earliest generations of lipid-based nanoparticles. Unlike LNPs, which are primarily used for nucleic acid delivery, liposomes can encapsulate a wider range of biomolecules, including drugs and proteins. They vary in size, with types such as small unilamellar vesicles (SUVs) and large multilamellar vesicles (MLVs) [3]. In this review, we primarily focus on the application of saccharide cryoprotectants in the freezing or lyophilization processes of LNPs encapsulating gene drugs. Additionally, representative studies on liposomes encapsulating small drug molecules and employing saccharides as cryoprotectants are included to provide a deeper context for discussion.

LNPs are widely used as nonviral drug delivery vectors, serving as "protective shields" for nucleic acids and transporting them into the cytoplasm to exert their effects [4–6]. Compared with viral vectors, LNPs offer advantages such as low cytotoxicity, low immunogenicity, and low mutagenicity [7–9]. A major milestone in LNP clinical translation was achieved when the first LNP-delivered siRNA drug, Onpattro®, for polyneuropathy caused by transthyretin amyloidosis was approved by the US Food and Drug Administration (FDA) in 2018 [10]. The LNP-delivered mRNA vaccines Comirnaty® and Spikevax® played crucial roles in controlling the COVID-19 pandemic in Europe and the United States, further underscoring the potential of LNPs in nucleic acid therapy and vaccine development [2,11–13]. However, the storage stability of LNP-mRNA drugs remains a challenge. LNP-mRNA nanoparticles are prone to aggregation, fusion, or drug leakage, necessitating storage and transport at low temperatures, which increases costs when they are stored in aqueous suspensions [14,15].

The Comirnaty® (BioNTech, Mainz, Germany; Pfizer, New York, NY, USA) and Spikevax® (Moderna, Princeton, NJ, USA) vaccines are currently stored in frozen form, with sucrose serving as a cryoprotective agent (cryoprotectant). Lyophilization, or freeze-drying, is a highly effective method for preserving the integrity and structure of LNP-mRNA formulations [16]. This process involves multistep stages of freezing and drying at low temperatures, which subject mRNAs and their nucleic acid cargo (LNPs) to various stresses, including low-temperature, freezing, and drying stresses (Figure 1). These stresses—such as crystallization and vacuum dehydration—can damage LNPs and their encapsulated mRNAs, thereby compromising the stability of the formulation [16–20]. To mitigate this effect, the addition of suitable cryoprotectants is essential to protect both the nucleic acid drugs and the LNP-mRNA structure during lyophilization.

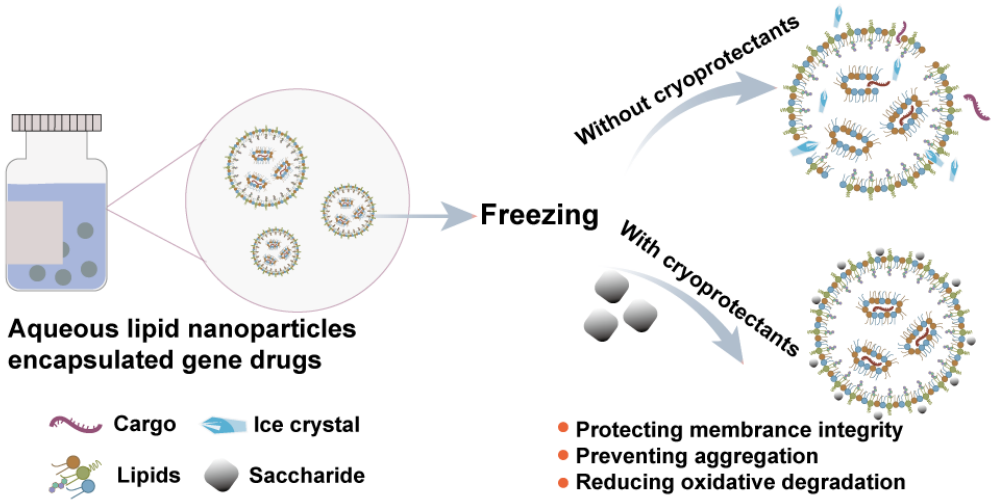

Figure 1. Diagram of the lyophilization of drug-loaded lipid nanoparticles with and without the addition of cryoprotectants.

Saccharides are the most commonly used cryoprotectants [21–25]. The type and concentration of the cryoprotectant, along with freezing conditions such as the cooling rate and ice nucleation method, significantly influence the success of the lyophilization process. Selecting the correct cryoprotectant is therefore critical for maintaining the structural integrity of both LNPs and the nucleic acids that are delivered. This review discusses the various types and characteristics of commonly used saccharide cryoprotectants, the mechanisms by which they protect LNPs-RNAs, and their applications in LNPs, providing guidance for choosing the most suitable cryoprotectants for the lyophilization of LNP-RNA formulations.

2. Types and Characteristics of Commonly Used Saccharide Cryoprotectants

Saccharides are carbohydrates that are widely distributed in nature and are strongly associated with animal and plant metabolism as fuel sources as well as structural components in plants [26]. Saccharides could be used as cryoprotectants in the preparation of mRNA-LNPs because of their special structure. The saccharides that have been evaluated for the freeze-drying of LNPs include monosaccharides, disaccharides, polysaccharides, and sugar alcohol [27]. The chemical structures of some representative saccharides used in the preparation process of frozen or lyophilized mRNA-LNPs are shown in Figure 2. Sugars can be divided into reducing sugars and nonreducing sugars based on whether they contain reducing groups (aldehyde or ketone groups) (Table 1).

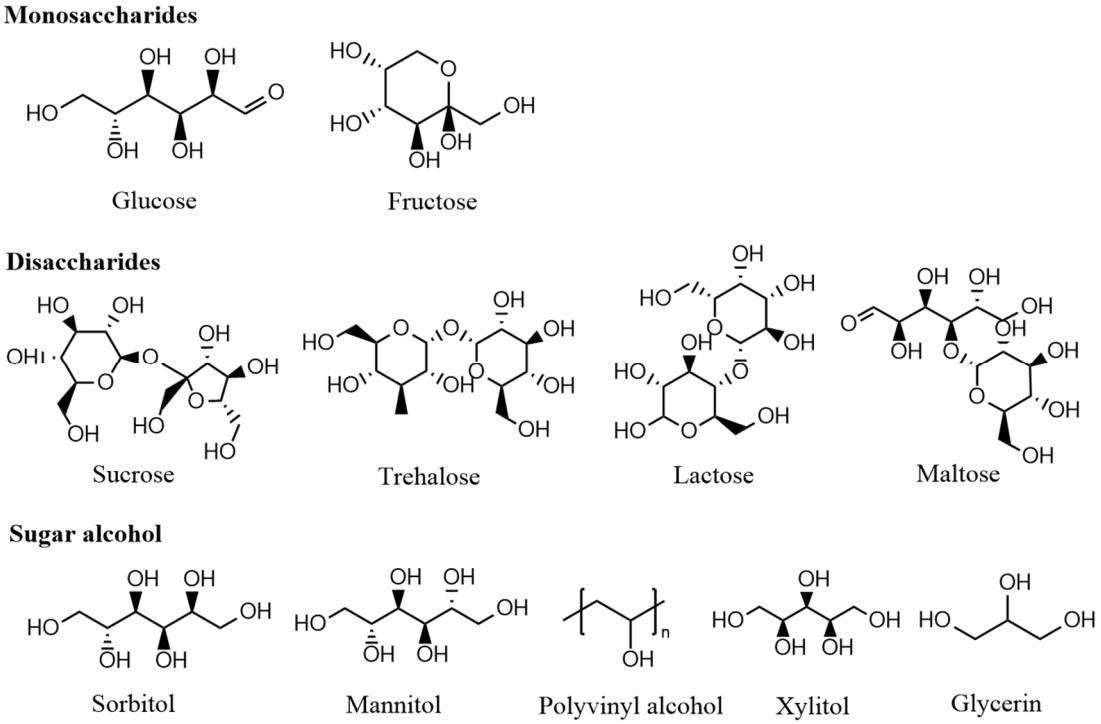

Figure 2. Chemical structures of commonly used saccharide cryoprotectants.

Table 1. Saccharide cryoprotectants for mRNA-LNPs.

Categories	Saccharide Cryoprotectants	Features	References
Non-reducing sugar	Sucrose, trehalose	Stable, high glass transition temperature	[28–31]
Reducing sugar	Glucose, fructose, lactose, maltose	The structure contains reducing groups, such as free aldehyde or free ketone groups, which are highly reducing, well soluble, and stable	[28,32,33]
Sugar alcohol	Sorbitol, mannitol, glycerol, xylitol, polyvinyl alcohol	Monosaccharide derivatives, resistant to heat, acid and alkali	[34]

Monosaccharides are the simplest form of carbohydrate and consist of a single unit that contains a carbon chain of three to six carbons [35,36]. The main function of monosaccharides is to produce and store energy in nature. Studies have demonstrated that monosaccharides, such as glucose [33,37,38], fructose [37,39,40], and mannose [37,39,41–44], can be used as cryoprotectants in the freezing or lyophilization of nanomaterials (Figure 2).

Disaccharides are sugar molecules formed by the combination of two monosaccharides via glycosidic linkages [45]. For example, sucrose is created by linking one glucose and one fructose molecule, and it is a commonly used material as a table sugar worldwide. Some studies have indicated that disaccharides can also be used as important cryoprotectants [46], including sucrose [30,47–51], trehalose [30,48–54], lactose [48], and maltose [48–51] (Figure 2). Zhao et al. demonstrated that disaccharides, such as sucrose and trehalose, performed better than monosaccharides in maintaining the stability of frozen or lyophilized LNPs because they can cure in the amorphous state and form more hydrogen bonds with LNPs [31].

Polysaccharides are the most abundant naturally occurring macromolecular polymers, with a degree of polymerization (DP) of up to approximately 10 consisting of monosaccharide monomers linked by glycosidic

bonds. Raffinose is a polysaccharide commonly used to maintain the structure of nanoparticles during freezing or lyophilization processes [55,56].

Sugar alcohols, also known as polyhydric alcohols (polyols), are carbohydrates and natural sugar substitutes as well as food additives. Compared with the corresponding sugars, sugar alcohols have an additional hydroxyl group and are therefore designated as polyols, polyalcohols, or polyhydric alcohols. Mannitol, sorbitol, galactitol, and glucitol are the main sugar alcohols and have been studied as cryoprotectants in this field (Figure 2) [57–59].

3. Mechanism of RNA-LNP Protection by Saccharide Cryoprotectants during Lyophilization

3.1. Lyophilization Process

Lyophilization is a dehydration process involving vacuum drying in a low-temperature environment (Figure 3) [60,61]. It is widely used to improve the long-term stability of thermally unstable or complex drugs, such as proteins, vaccines, and nanoparticles, while preserving their biological activity [18]. However, even though it involves relatively few processing steps, the lyophilization process is complex, with different pressures applied at each stage: freezing, primary drying, and secondary drying. Each step, including freezing, sublimation, and reconstitution, can affect drug efficacy, and LNPs are particularly susceptible to damage [62].

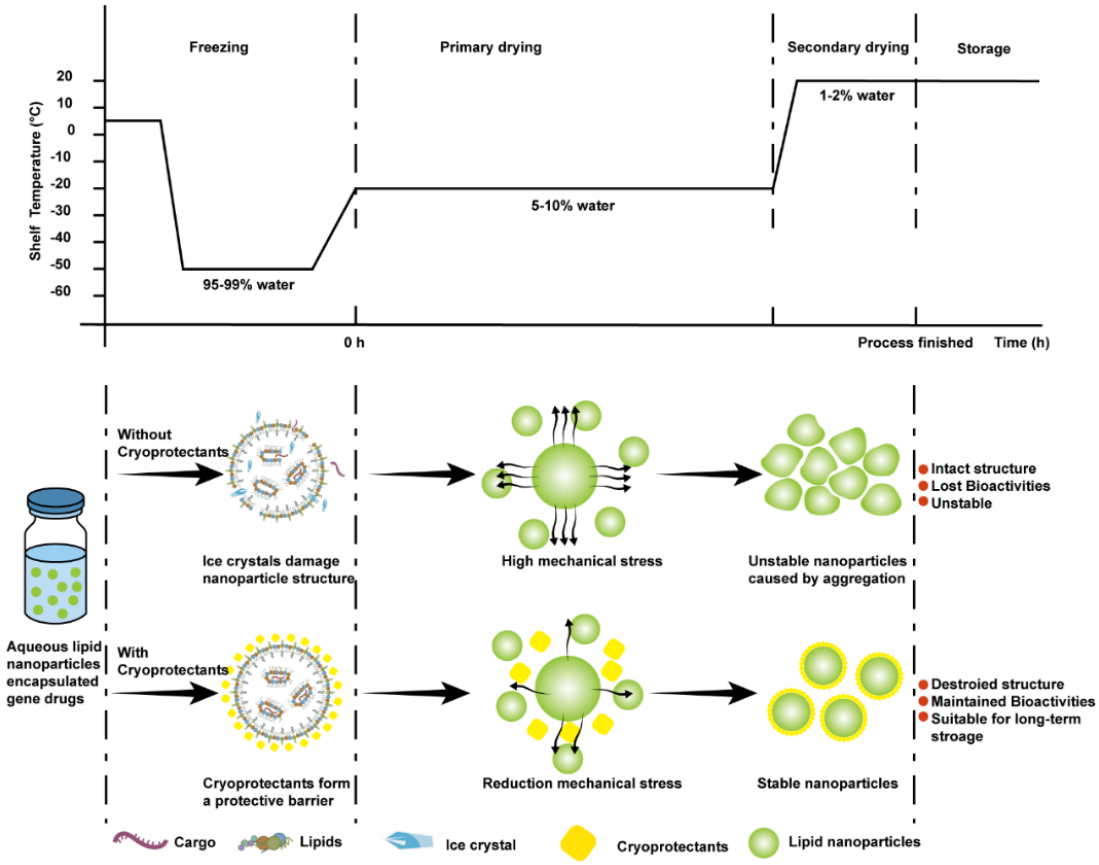

Figure 3. Freeze-drying process with or without cryoprotectants.

Freezing, although the shortest step in the lyophilization process, has a significant effect on the quality and stability of freeze-dried drugs [63,64]. Ultrarapid cooling, such as immersing LNP-mRNA in liquid nitrogen, results in the formation of fine ice crystals and a uniform distribution of protective agents, which may reduce disruption of the bilayer structure of the LNP-mRNAs [65]. In contrast, a slow freezing rate minimizes supercooling and osmotic pressure, reducing ice crystal formation inside the LNPs and preventing drug leakage [66,67]. In addition, LNPs are prone to aggregation and fusion during the freezing step (as shown in Figure 3), so protective agents (cryoprotectants) are usually added to reduce the damage caused by low temperatures [68].

There are two types of cryoprotectants: non-penetrating cryoprotectants and penetrated cryoprotectants (Figure 4). Non-penetrating cryoprotectants, such as saccharides, stabilize LNP membranes by interacting with the polar head, reducing lipid—water contact. Penetrating cryoprotectants penetrate the membrane, replacing

water to inhibit ice crystal formation. Non-penetrating cryoprotectants generally cause less damage at the same dose and are preferred for lyophilization, as they prevent osmotic shock throughout the entire lyophilization process and the subsequent rehydration process [21,69–71]. Saccharides are the most commonly used non-penetrating cryoprotectants for LNPs. There are two main hypotheses concerning the underlying cryoprotective mechanisms of saccharide treatment based on previous experiments: water replacement and vitrification [69,72].

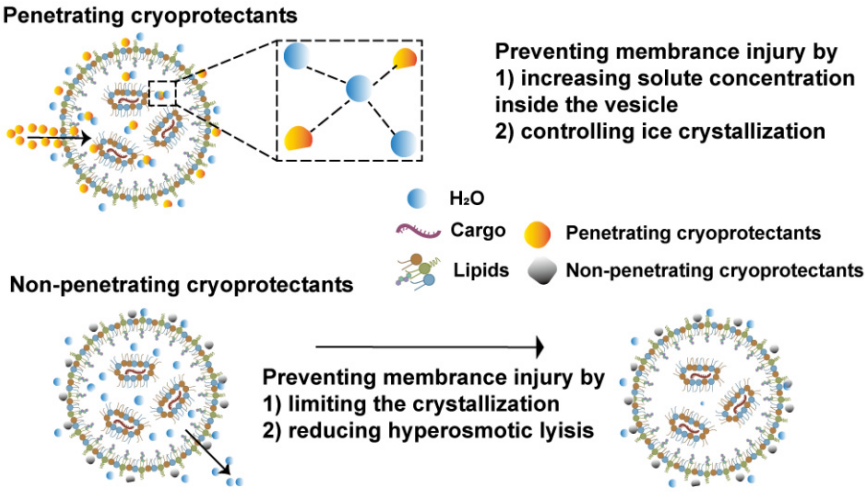

Figure 4. The protection effectiveness of non-penetrated cryoprotectants and penetrated cryoprotectants (e.g., saccharides) for protection of the integrity of LNP-delivered gene drugs.

3.2. The Water Replacement Hypothesis

The water replacement hypothesis proposes that saccharide-based cryoprotectants, which are rich in hydroxyl groups, can substitute for water molecules on the surface of LNPs. In this hypothesis, saccharides interact with lipid head groups, mimicking the hydration effect and maintaining bilayer stability similar to that of a fully hydrated system [54]. During dehydration, these saccharides preserve the structural integrity of the cell membrane and protect the lipids in LNPs [73]. During the freezing process, saccharides reduce the interaction between phospholipids and water, maintain the spatial distance between phospholipid head groups, and lower van der Waals forces between hydrocarbon chains (Figure 5). This process reduces the phospholipid bilayer transition temperature (Tm), improving the structural stability of LNPs. Upon rehydration, water molecules quickly replace saccharides, allowing the bilayer to reform before leakage occurs and preventing drug leakage. This mechanism supports the structural stability of LNP formulations containing saccharides during the lyophilization process. Studies using molecular dynamics (MD) simulations, nuclear magnetic resonance (NMR), and fluorescence microscopy observations have provided evidence for the water replacement hypothesis [74].

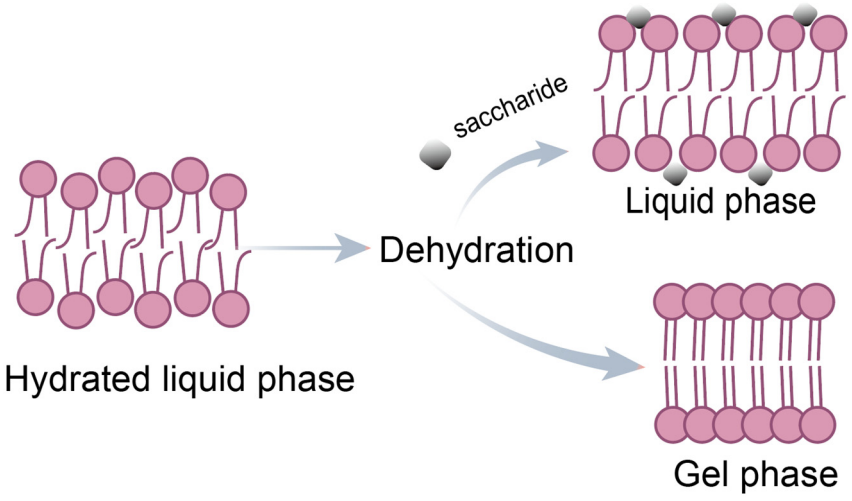

Figure 5. Effect of sugar on the dehydration of phospholipid bilayers.

3.3. The Vitrification Hypothesis

The vitrification hypothesis proposes that saccharide-based cryoprotectants affect the glass transition temperature (Tg) of LNPs, which is crucial for optimizing the lyophilization process [75–77]. According to this theory, saccharides can form a glassy state with extremely high viscosity, a low molecular diffusion coefficient, and resistance to crystallization [78,79]. In this state, the saccharide acts as a viscous barrier around the LNPs, reducing their movement and preventing adhesion between particles. This glassy state also protects the lipid bilayer from mechanical damage caused by ice crystals, preventing vesicle fusion [80,81].

Importantly, the vitrification and water replacement hypotheses act synergistically to maintain the stability of LNPs at low temperatures, complementing each other rather than acting independently [64,82,83]. Based on these two hypotheses, an ideal saccharide-based cryoprotectant should possess a high glass transition temperature, a low crystallization rate, low moisture absorption and the ability to form hydrogen bonds.

4. The Application of Saccharide-Based Cryoprotectants in Frozen or Lyophilized LNP-Delivered Gene Therapies

Currently, saccharide-based cryoprotectants have been successfully applied in the lyophilization process of various gene therapies delivered by LNPs [84–87], including the two FDA-approved frozen mRNA vaccines Comirnaty[@] and Spikevax[@]. The type and concentration of saccharides play crucial roles in preserving the redispersibility and stability of LNP-delivered gene therapies [88]. In the following sections, we review representative studies that examine how different types and concentrations of saccharides influence the structure and stability of LNP-delivered gene therapies. The saccharide-based cryoprotectants reported in the relevant literature are summarized in Table 2 [28–33,59,89]. This analysis aimed to identify the saccharides most effective at maintaining the integrity and stability of these therapeutic systems.

Sucrose, a nonreducing disaccharide with a high glass transition temperature, is one of the most commonly used cryoprotectants [21,28,67,90–92]. Sucrose is included in the FDA-approved frozen mRNA vaccines Comirnaty[@] and Spikevax[@] to maintain stability in low-temperature environments, enabling Comirnaty[@] to be stored at −70 °C and mRNA-1273 at −20 °C for more than 6 months [47]. However, the ultralow-temperature storage environment greatly limits the distribution and transportation of vaccines in developing countries and regions. Lamoot et al. developed lyophilized LNP-mRNA using sucrose (20% *w*/*v*) as a cryoprotectant and reported that lyophilization had a minimal effect on the size and zeta potential of LNP-mRNA, allowing for successful reconstitution in aqueous media [28].

Trehalose, another commonly used nonreducing disaccharide, is known for its ability to maintain the structural integrity of LNPs and enhance their resistance to drying [93,94]. Compared with other saccharides, trehalose has key advantages, including a high glass transition temperature, low hygroscopicity, lack of intramolecular hydrogen bonds, and flexibility in forming hydrogen bonds with nanoparticles during lyophilization [95]. Amis et al. compared the effects of cryoprotectants, including pectin, glycine, mannitol, polyvinylpyrrolidone (PVP), sorbitol, and trehalose, on the particle size distribution of solid lipid nanoparticles (SLNs) after freeze–thaw cycles and lyophilization [29]. Compared with the other cryoprotectants, the SLNs containing 20% (*w*/*w*) trehalose presented the smallest particle size (approximately 332.5 nm) and polydispersity index (PI mean of 0.0061). Therefore, 20% (*w*/*w*) trehalose was selected as the optimal cryoprotectant condition in this study [29]. Khan et al. also developed lyophilized nanostructured lipid carriers using trehalose as a cryoprotectant, and the optimized freeze-dried formulations demonstrated stable storage for 6 months under refrigeration conditions (5 °C ± 3 °C) [89].

Ball et al. evaluated the effects of various concentrations of sucrose and trehalose (0%, 1%, 5%, 10%, and 20% *w*/*v*) on the long-term stability of lyophilized LNPs encapsulating siRNA. The results showed that a 20% concentration of either sucrose or trehalose achieved gene silencing effects in HeLa cells comparable to those in fresh samples after lyophilization and reconstitution [30]. Similarly, Zhao et al. reported that lipid-like frozen nanoparticles (LLNs) with 5% (*w*/*v*) sucrose or trehalose preserved mRNA delivery efficiency for more than three months under liquid nitrogen storage [31]. While sucrose and trehalose each offer distinct advantages as cryoprotectants, distinguishing between them in terms of overall effectiveness in maintaining the integrity and stability of LNP-delivered gene therapies remains challenging [96–98]. Overall, sucrose and trehalose have their own advantages and are difficult to distinguish when they are used as cryoprotectants.

Table 2. The application of saccharide-based cryoprotectants in different LNP-delivered gene therapies and regenerative medicine.

Formulation	Cargo	Tested Cryoprotectants	The Optimal Cryoprotectants	Status	Reference
S-Ac7-Dog or S-Ac7-DHDa, respectively, DSPC, cholesterol and DMG-PEG_{2000}	mRNA	Glucose, fructose, mannitol, sucrose, lactose, trehalose	20% (*w*/*v*) sucrose	Freeze-dried	[28]
Stearic acid	Progesterone	Glucan, glycine, mannitol, PVP 40, sorbitol, and trehalose	20% (*w*/*w*) trehalose	Freeze-dried	[29]
Lipidoids, DSPC, cholesterol, DMG-PEG_{2000}	siRNA	sucrose, trehalose	20% (*w*/*v*) sucrose or trehalose	Freeze-dried	[30]
TT3, DOPE, cholesterol, DMG-PEG_{2000}	mRNA	Sucrose, trehalose, and mannitol	5% (*w*/*v*) sucrose or trehalose	Freeze	[31]
HSPC, DPPG	Nifedipine	Glucose, fructose, maltose and sucrose	100 mg glucose, fructose, maltose, and sucrose	Freeze-dried	[32]
Compritol® 888 ATO	None	Glucose, mannitol	1% (*w*/*v*) glucose	Freeze-dried	[33]
Sodium deoxycholate monohydrate, Super Refined Brij O2	Zein, gliadin	Glucose, mannose, sucrose, trehalose, and mannitol	Mannose	Freeze-dried	[39]
TT3, Dlin-MC3-DMA, DOPE, Cholesterol, and DMG-PEG_{2k}	repRNA	Sucrose	10% (*w*/*v*) sucrose (stable for 30 days at −20 °C)	Freeze-dried	[47]
Ionizable lipid, DSPC, cholesterol, and PEG_{2000}-C-DMA	mRNA	Sucrose, maltose	With 10% (*w*/*v*) sucrose and 10% (*w*/*v*) maltose.	Freeze-dried	[50]
Glyceryl monostearate	Celecoxib	Mannitol, sorbitol, glycerol, glucose, fructose, mannose, trehalose, maltose, sucrose, lactose	Trehalose	Freeze-dried	[51]
C12-200, DOPE, cholesterol, C14-PEG_{2000}	pDNA	Sucrose, trehalose, sorbitol	20% (*w*/*v*) trehalose	Freeze-dried	[52]
HSPC, DMPG	Griseofulvin	Maltose, sucrose, xylose, mannose, fructose, lactose, raffinose	Mannose, fructose, lactose, raffinose	Freeze-dried	[55]
Glyceryl behenate, egg-phosphatidylcholine, poloxamer 188	Sorafenib	Trehalose, mannitol	15% (*w*/*v*) mannitol	Freeze-dried	[58]
DMG-PEG_{2000}, ionizable lipid A/B, cholesterol, DOPE	mRNA	Leucine alone or in combination with mannitol	Combination of leucine and mannitol	Dry powder	[59]
Compritol ATO 888® and oleic acid	Verapamil	Mannitol, fructose, sucrose, lactose, and trehalose	Trehalose (stable for 6 months at refrigerated condition)	Freeze-dried	[89]

Researchers have also compared the protective effects of reducing and nonreducing saccharides on lyophilized LNPs. Ohshima et al. reported that a nifedipine-LNP suspension supplemented with glucose, fructose, maltose, or sucrose before lyophilization could inhibit the aggregation of nanoparticles upon reconstitution [32]. Similarly, Santonocito et al. reported the lyophilization treatment of a SLN aqueous solution and reported that the addition of 1% (*w*/*v*) glucose as a cryoprotectant provided the best protective effect during the lyophilization process [33]. However, studies by Horn et al. revealed that LNPs with 20% (*w*/*v*) mannitol were structurally unstable after freeze–thaw cycles, possibly because of mannitol crystallization during freezing [99]. Additionally, Wolkers et al. reported that LNPs supplemented with fructose and glucose resulted in a collapsed cake upon lyophilization, probably due to the lower glass transition temperature of monosaccharides than disaccharides, leading to structural instability [100].

Some studies have also explored the protective potential of sugar alcohols, such as xylitol and glycerol, for maintaining the stability of LNP-delivered gene therapies. Kamiya et al. demonstrated that LNPs supplemented with xylitol can minimize the aggregation of nanoparticles after extensive lyophilization [34]. Glycerol, a simple polyol, is commonly used as a low-temperature protectant in lyophilized LNP formulations due to its relatively low toxicity at high concentrations compared with other cryoprotectants, which helps to protect lipid membranes during dehydration [101]. However, research on the specific advantages of glycerol for cryoprotection in LNPs is

limited. In addition, few studies have addressed the mechanism by which sugar alcohols prevent nanoparticle aggregation during lyophilization [102].

The saccharide concentration also significantly affects the stabilizing effect on LNP formulations, in addition to the key influence of saccharide type on LNP stability during lyophilization [21,103]. Ball et al. reported that LNP formulations consisting of lipidoid, distearoylphosphatidylcholine (DSPC), cholesterol, and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG_{2000}) (molar ratio 50:10:38.5:1.5) encapsulating siRNA exhibited optimal performance in terms of gene silencing, siRNA retention, particle size, and monodispersity when 20% (*w*/*v*) sucrose or trehalose was supplemented [30]. Conversely, Zhao et al. reported that a lower cryoprotectant concentration of 5% (*w*/*v*) sucrose or trehalose was optimal for delivering mRNA using a lipid-like nanoparticle (LLN) formulation containing TT3, dioleoylphosphatidylethanolamine (DOPE), cholesterol, and DMG-PEG_{2000} (molar ratio 20:30:40:0.75) [31]. These studies illustrate that the optimal concentration of cryoprotectants is not fixed; rather, it depends on the lipid components and lipid ratios within the LNP formulations, with the most commonly used concentration ranging from 1% to 20%.

In summary, sucrose and trehalose are the most commonly used cryoprotectants suitable for maintaining LNP stability during lyophilization. Disaccharides such as sucrose and trehalose have proven to be more effective than monosaccharides and sugar alcohols at preserving the original structure and stability of LNPs, which encapsulate not only regenerative medicines but also gene therapies throughout the lyophilization process. Generally, sucrose is significantly cheaper than trehalose at both bulk and retail prices [104]. For pharmaceutical-grade or high-purity for large quantities, which is used in more specialized applications such as lyophilization, sucrose can cost $1 to $3 USD per kilogram, while trehalose can cost $30 to $80 USD per kilogram, or even higher, depending on the supplier. This makes sucrose the preferred cryoprotectant for large-scale manufacturing applications where cost-effectiveness is a priority, especially in industries like food production and biotechnology. Despite the higher price of trehalose, it is increasingly being used in more specialized applications where its enhanced protective properties justify the cost [105].

The combined use of sucrose or trehalose with other cryoprotectants is also an ideal option for maintaining LNP stability. Muramatsu et al. demonstrated that the combination of 10% sucrose and 10% maltose (*w*/*v*) as cryoprotectants can preserve the physicochemical properties of lyophilized mRNA-LNPs for 12 weeks at room temperature and for at least 24 weeks at 4 °C without significant changes [50]. The appropriate concentration and choice of cryoprotectants must be tailored to the specific LNP composition, highlighting the need for precise optimization.

5. Technical and Regulatory Challenges in Scaling Cryoprotectants for Large-Scale Clinical Production

In preclinical research, optimizing the selection, concentration, and combinations of saccharide-based cryoprotectants has been a major focus in improving the stability of LNP-encapsulated gene drugs during cryopreservation and lyophilization. These efforts have significantly advanced our understanding of how cryoprotectants can be tailored to enhance the efficacy and shelf life of gene therapy formulations. However, translating these findings from laboratory-scale studies to large-scale clinical production introduces a new set of challenges, particularly in the areas of technical implementation and regulatory compliance [106,107].

5.1. Technical Challenges in the Clinical Scale-Up of Cryoprotectants

Existing cryopreservation protocols developed for small-scale laboratory settings may not be directly applicable to large-scale clinical production [108]. In particular, freezing and thawing rates must be optimized for large batches to avoid ice formation and preserve the stability of the cryopreserved product [23]. The challenge becomes more pronounced when considering the high concentrations of cryoprotectants often required. At elevated levels, cryoprotectants (especially saccharides like sucrose and trehalose) can cause osmotic stress or even toxicity [105,109], potentially compromising the integrity of LNPs. Furthermore, the batch-to-batch variability in cryoprotectant efficacy, which can arise during scale-up, may lead to inconsistent product quality and affect clinical outcomes. Ensuring product consistency and quality control at scale requires careful management of cryoprotectant performance across multiple manufacturing batches. The availability and cost of some cryoprotectants, such as trehalose, also represent challenges for scaling up, especially in low-resource settings or developing countries [105].

5.2. Regulatory Challenges in Safety and Toxicity Assessments

The use of cryoprotectants in clinical-grade formulations must adhere to stringent regulatory guidelines from authorities such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Common

saccharide-based cryoprotectants such as sucrose, trehalose, mannitol, glucose, and lactose are generally regarded as safe and are widely used in FDA-approved and EMA-approved drug formulations, particularly in lyophilization and cryopreservation processes [110]. Sucorse has been successfully applied in the two FDA-approved frozen mRNA vaccines Comirnaty@ and Spikevax@ [3].

Transitioning from preclinical formulations to clinical production also requires strict adherence to Good Manufacturing Practices (GMP) standards. These regulations ensure that cryoprotectants do not introduce contaminants and that batch-to-batch quality is consistent and reproducible. Regulatory authorities, such as the FDA and EMA, also require comprehensive data on the shelf life and long-term stability of cryoprotectant-treated gene drugs. This data must demonstrate that cryoprotectants do not interfere with the therapeutic efficacy of the gene therapy product over extended periods.

For gene therapy products encapsulated in LNPs, the regulatory framework for both the LNP carrier and the cryoprotectant needs to be well-defined. This includes understanding how cryopreservation may impact both the lipid nanoparticles and the therapeutic payload, which requires close collaboration with regulatory agencies to ensure that cryoprotectants meet specific regulatory guidelines.

6. Conclusions and Prospects

Cryoprotectants are essential ingredients in the preparation of frozen or lyophilized LNPs for use in gene therapies or regenerative medicine, as they help maintain structural integrity and stability during freezing or lyophilization [88]. They achieve this by lowering the glass transition temperature of the RNA-LNP solution and/or by replacing water molecules in the RNA-LNP solution, thus inhibiting crystal growth. Disaccharides such as sucrose and trehalose have proven to be the most effective saccharide-based cryoprotectants. The optimal saccharide concentration varies depending on the LNP formulation, including the different lipid types.

The synergistic effects of complex cryoprotectants, as well as combinations of cryoprotectants and optimized freezing or lyophilization technologies [111] (e.g., shelf freeze-drying and spray freeze-drying methods), may yield excellent preservation efficiency, but further research is needed to confirm this hypothesis.

Recent advances have shown growing potential in exploring alternative cryoprotectants, such as peptide-based cryoprotectants (e.g., antifreeze proteins, peptide amphiphiles) and polymer-based cryoprotectants (e.g., polymeric cryoprotectants, block copolymer cryoprotectants) [112,113], in addition to the traditional saccharide-based options for LNP-encapsulated gene drugs. These peptide- and polymer-based cryoprotectants offer several advantages, including the ability to form a protective layer around nanoparticles that prevents aggregation and enhances stability during freezing and lyophilization [114,115].

The use of these alternatives could also help reduce reliance on high concentrations of saccharides, which can sometimes lead to osmotic stress or other biological issues. Furthermore, peptide- and polymer-based cryoprotectants are highly scalable and cost-effective, offering significant potential for industrial applications [116]. These developments in cryoprotectant technology could have significant implications for the preservation of LNPs used in gene therapy and regenerative medicine, providing more efficient, sustainable, and biocompatible alternatives to current cryopreservation techniques.

In addition to cryoprotectant optimization, the introduction of novel lyophilization techniques for cryoprotection of LNP-delivered gene drugs has shown great promise in improving lyophilization yield, nanoparticle stability, and biological activity [91]. For example, Higuchi et al. have schematically illustrated several freeze-drying methods that may improve the scalability and cost-effectiveness of mRNA vaccine production. These techniques could contribute to challenges in the global distribution and storage of cryoprotected mRNA vaccines [16].

Advancements in both cryoprotectants and lyophilization methods for mRNA-LNPs could also be applied to other gene therapies, such as gene editing (e.g., CRISPR-Cas9), stem cell therapies, and protein-based therapeutics. Given the rapidly expanding field of regenerative medicine, cryopreservation techniques using novel cryoprotectants could facilitate the long-term storage and transport of complex biologics, thus enhancing the accessibility of cell therapies and gene therapies for clinical use.

Overall, there is an urgent need for a universally applicable cryoprotectant formulation for various LNP types [117]. The health hazards and applicability of LNP drugs should also be considered when developing novel cryoprotectants. A comprehensive understanding of the factors influencing long-term LNP stability is crucial for extending shelf-life, ensuring safety, reducing production costs, and facilitating clinical translation. The optimization of cryoprotectant formulations will be central to these efforts, enabling a wider application of LNP-based drug delivery systems in medicine, especially regenerative medicine.

Author Contributions

W.L.: writing of the original draft, conceptualization, data curation. T.W.: writing, reviewing, edition, methodology, funding acquisition. J.C.: visualization. M.G.: validation, supervision. L.L.: validation, supervision. A.H.: writing, review, editing, supervision, funding acquisition, conceptualization. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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