Physical stabilization of DNA-based therapeutics

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The development of non-viral vectors for gene delivery has primarily focused on improving the efficiency of gene transfer *in vivo*. Although there is clearly a need to improve delivery efficiency, studies also indicate that the physical stability of non-viral vectors is not nearly adequate for a marketable pharmaceutical product. Here, we describe the different strategies that have been used to enhance stability and discuss the mechanisms by which prolonged stabilization (>2 years) might be achieved.

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▼ The ability to deliver genes to cells and tissues *in vivo* offers the potential to treat many hereditary diseases that are currently considered incurable (e.g. some cancers, cystic fibrosis)[1–3]. Considering the tremendous promise of DNA delivery technology and the extensive genetic information that is now available from the Human Genome Project, it is not surprising that some tout gene therapy as the next revolution in medicine. Along with the enormous expectations for applications of this technology, the pressure to obtain positive clinical results has led to an empirical approach to achieving successful gene delivery.

Not surprisingly, most of the clinical trials to date have used viruses that are known to deliver genes efficiently in cell culture and animal models[1]. However, viral vectors can also elicit a potent immune reaction that has been implicated in the death of patients enrolled in clinical studies[4]. In addition, problems associated with limited viral capacity, difficulty in targeting and the potential for mutation and reproduction have stimulated an interest in developing synthetic, non-immunogenic vectors for gene therapy[5]. Although contemporary non-viral vectors are relatively inefficient compared with viruses, it should be appreciated that non-viral vectors have been under development for only a decade, whereas viruses have been evolving gene delivery mechanisms for billions of years.

Significant progress towards the development of clinically effective non-viral gene therapy continues to be achieved. To date, cationic lipid-based vectors have been the most extensively studied, and clinical trials have clearly shown that lipid–DNA complexes are safe and non-immunogenic[6]. Furthermore, the lack of a specific immune response allows synthetic vectors to be administered in repetitive doses that have been shown to increase and prolong therapeutic gene expression[3,7]. Despite these advantages, non-viral gene delivery systems have yet to demonstrate therapeutic levels of gene expression in human clinical trials. These disappointing clinical results have justified the predominant focus in non-viral gene therapy research, on improving transfection efficiency. However, crucial pharmaceutical aspects (e.g. stability) of non-viral vector development have been virtually ignored.

To realize the enormous potential of non-viral gene therapy, vectors with consistent physical and chemical properties will need to be manufactured on an industrial scale. Currently, non-viral vectors are made in relatively small batches by mixing a solution of DNA with a suspension of a cationic agent (e.g. lipid, polymer). The DNA and polycation interact electrostatically to form complexes that are ultimately used for gene delivery. However, this method of preparation results in a highly heterogeneous suspension of particles possessing a wide range of charge ratios and sizes[8–11]. Furthermore, the particle size, ζ potential and other biophysical characteristics that are known to affect gene delivery

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efficiency can themselves be affected by many factors including the mixing protocol, buffer components and complexation time[12–14]. These issues complicate manufacturing scale-up and will clearly need to be addressed if non-viral gene delivery proves to be clinically effective.

Assuming that adequate manufacturing procedures can be developed, it is imperative that the biophysical properties and gene delivery efficiency of non-viral vectors be preserved until the product is administered in the clinic. Stability is a requirement even for the relatively small-scale preparations currently being used for phase-I studies. Clearly, instability of the preparation after production will adversely affect quality control and transfection rates, which are known to plague clinical gene therapy trials. Although other factors can dramatically alter the efficiency of gene transfer (e.g. route of administration, target tissue, rate of cell division, chemical integrity, polycation formulation), in this article, we focus on the physical stability of non-viral vectors and the maintenance of particle characteristics during processing and storage. These obstacles must be overcome if the advantages of non-viral gene therapy are to be realized.

Liquid formulations

It is well known that aqueous suspensions of non-viral vectors have a tendency to aggregate over time[15,16]. Early clinical trials attempted to circumvent this problem by preparing complexes at the bedside, immediately before injection[6,17]. Clearly, this method of sample preparation and administration is not practical and leads to significant variations in product quality and gene delivery efficiency. This problem has stimulated an interest in developing 'stable' synthetic vectors that resist aggregation. Although more recent formulations claim to have improved stability, their shelf-life is typically measured in hours or days rather than the 18-month–2-year timeframe required for a marketable pharmaceutical product[7,18,19].

Other studies have used different approaches in an attempt to prevent aggregation of non-viral vectors. For example, Caplen and colleagues[20] diluted complexes with an alkaline solution that was intended to enhance stability by an undescribed mechanism. Using a different approach, Hofland and coworkers[21] and Gao and Huang[22] used sucrose density-gradients to separate the heterogeneous population of particles and to isolate a stable fraction of lipid–DNA complexes. These stable fractions could be stored under refrigerated conditions for three months without detectable losses in transfection rates. It has subsequently been shown that these complexes deliver genes effectively *in vivo*[23]. Although this separation technique can be applied to research-scale projects, it is doubtful that sucrose density fractionation would be practical for bulk manufacturing.

In addition to isolating a stable fraction of complexes, the studies with sucrose gradients described above indicate that lipid–DNA complexes are more stable if they are isolated from the bulk suspension. Considering that preparations of lipid–DNA complexes typically contain free (i.e. uncomplexed) liposomes and DNA, these findings suggest that interactions between complexes and free components can result in decreased rates of gene delivery. This is not surprising if we consider the impact that the incorporation of additional polynucleotides and/or the fusion of excess cationic liposomes could have on a vector's charge, size and lipid–DNA interactions.

To enhance stability, it should be possible to develop methods of preventing interactions among components within a single suspension. In fact, Hong and colleagues[24] and Eastman and coworkers[25] used polyethyleneglycol–lipid (PEG–lipid) conjugates to prevent aggregation of non-viral vectors and sterically stabilize particles in aqueous suspensions. Studies have also used PEG derivatized to anionic peptides to coat cationic particles and to reduce aggregation with serum components[18,26,27]. Although the use of PEGylated components appears to reduce the aggregation of non-viral vectors, the steric stabilization is also known to curtail interactions with cells and to interfere with cellular processing[10,28]. Therefore, nonviral vectors containing PEGylated components typically have lower transfection activities than their non-PEGylated counterparts[28]. Curiously, the inhibitory effect of PEG on *in vivo* transfection was attenuated after storage at 4°C for one month[24].

The use of condensing polyamines has also been shown to increase the stability of non-viral vectors and to prevent aggregation during storage[24,29]. Although it is doubtful that stabilization via condensation would be sufficient for prolonged storage as an aqueous preparation, studies with suspensions of poly[(2-dimethylamino)ethyl methacrylate]-based gene delivery systems claim that transfection rates were retained for 10 months at 20°C (Ref. 30). The increased stability of some of the systems described above is encouraging but storage stability studies typically neglect stresses that are commonly encountered during processing and/or shipping (e.g. agitation, freeze–thawing[31]). Considering that both of these stresses can significantly reduce the transfection rates of non-viral vectors[15,31], it is prudent to develop formulations that can resist shipping-induced damage.

Frozen formulations

The effect of freeze–thawing on DNA was first reported by Shikama more than 35 years ago[32]. This study showed that

the double helix of calf thymus DNA remained intact during freezing to temperatures as low as –192°C (Ref. 32). Several years later, Lyscov and Moshkovsky[33] described a mechanism of DNA degradation during freezing (cryolysis) that depended on the rate of cooling of the frozen sample. They suggested that cryolysis resulted from the formation of cracks within the ice during freezing and showed that these cracks were more prevalent in rapidly cooled samples[33]. Recently, very rapid cooling by immersion of samples into liquid nitrogen has been used to protect DNA from shear stress during homogenization[34].

However, Ando and colleagues[34] clearly showed that both sugars and EDTA were required to preserve supercoil content during freezing. Although it is not clear whether these excipients specifically protect against the cryolysis mechanism of degradation described by Lyscov and Moshkovsky, it is possible that glass formation, caused by the addition of lactose, inhibits the cracking that is implicated in DNA degradation[35]. The presence of EDTA might inhibit DNase[34], or it could chelate divalent cations (e.g. Fe^{2+}), which are known to facilitate the hydroxyl-radical formation involved in DNA degradation[36,37].

Complexation with cationic agents

Although the studies on DNA alone might be applicable to the freezing of naked DNA formulations for vaccines, it has been shown that complexing DNA with cationic agents greatly stabilizes polynucleotides during physical stress[38,39]. It is noteworthy that Densmore and coworkers[40] have shown that cationic agents differ greatly in their ability to protect DNA during aerosolization. These findings suggest that specific interactions with the polynucleotide chain might be necessary for optimal protection. The precise nature of these stabilizing interactions has yet to be investigated but elucidating these protective mechanisms is of obvious importance to the development of gene-based therapeutics in general, and particularly relevant to pulmonary delivery.

Studies on the physical stabilization of DNA-based therapeutics have mostly focused on the preservation of complexes that are known to function as non-viral vectors[41]. As described above, aqueous suspensions of non-viral vectors are acutely sensitive to agitation stress encountered during shipping[31]. One method to circumvent this problem would be to develop frozen formulations that are resistant to agitation-induced aggregation. If we consider the intricate associations between cationic agent and DNA within a complex that are required for gene delivery, it is not surprising that non-viral vectors are sensitive to freezing stress[15,31]. In addition to reductions in transfection activity, increases in particle size indicate that aggregation occurs during the freeze–thaw process[31,42,43]. Although this damage is extensive after a slow freeze–thaw cycle, rapid freezing and thawing results in minimal losses in transfection activity, and the maintenance of particle size[31,44].

These findings suggest that the increased time during which particles can diffuse during slow cooling promotes the aggregation of non-viral vectors that results in reduced transfection rates[31,41,43]. This mechanism of damage is consistent with the observation that rapid freezing by immersion in liquid nitrogen minimizes aggregation, presumably because there is not sufficient time for particles to collide in a liquid phase when samples are rapidly cooled. Furthermore, the fact that maintenance of particle size correlates strongly with the recovery of transfection rates indicates that aggregation during freezing represents the major mechanism of damage. It is worth noting that an increase in particle size can result from irreversible phenomena (e.g. lipid fusion) or from simple clumping[11]. The aggregation of some preparations is reversible (T.J. Anchordoquy *et al.*, unpublished) but it is not known whether dispersion of aggregates results in recovery of transfection activity. Although little attention has been paid to these different modes of aggregation, further characterization of these different assemblies is clearly warranted.

Role of cryoprotectants

The ability of 'cryoprotectants' to prevent aggregation of proteins and liposomes during freezing is well established[45–47]. The manifestation of using stabilizing excipients (i.e. the prevention of aggregation) is the same for both proteins and liposomes, but the protective mechanism appears to be different for macromolecules in solution (e.g. proteins) than for suspended particles (e.g. liposomes). In the case of proteins, aggregation typically involves some 'aggregation-competent' intermediate that is partially unfolded. Aggregation can, therefore, be prevented by using excipients to inhibit the formation of partially unfolded species[46]. The mechanism by which stabilizing excipients, typically sugars, attenuate protein unfolding during freezing was described by Timasheff and coworkers to explain solution stability and was termed preferential exclusion[48].

This thermodynamic mechanism involves the exclusion of solutes from the hydration shell of the protein and the subsequent stabilization of the proteins' native structures. Although a detailed discussion of preferential exclusion is beyond the scope of this article, it is important to point out that this stabilization mechanism derives from the inability of some solutes to access the surface of the protein (i.e. stabilizing solutes do not bind to the protein). By contrast, sugars are thought to stabilize liposomes by

interacting directly with the membrane surface during freezing[45,47]. The implication of a direct interaction in stabilization does not preclude preferential exclusion of the excipient overall, but it does suggest that the solute associated with the membrane surface is responsible for preserving vesicle integrity during freezing[45,47]. In this way, solute-induced stabilization of suspended particles is similar to the steric stabilization observed with PEGylated lipids[49].

The application of the stabilization mechanisms described above to the preservation of non-viral vectors has not been extensively studied. There have been several reports that sugars inhibit aggregation during freezing but the mechanism has yet to be definitively elucidated. Clearly, neither plasmid DNA nor cationic liposomes 'unfold' in a manner analogous to proteins, so it is difficult to invoke the preferential exclusion mechanism in the observed solute-induced maintenance of particle size during freezing. Instead, non-viral vector preparations are similar to liposomes in that they are a suspension of particles that can be sterically stabilized by the incorporation of PEGylated components[18,24,26,27,49]. It is thus plausible that the direct interaction mechanism described above for liposomes might also apply to non-viral vectors. However, much more sugar is required to prevent the aggregation of non-viral vectors than is needed to inhibit liposome fusion during freezing[43,45]. The relatively large quantities of excipients suggest that the maintenance of particle size is a result of nonspecific bulk characteristics of the formulation. We have suggested two mechanisms that might contribute to the observed retention of non-viral vector size during freezing – glass formation and particle isolation.

Glass formation

The formation of ice during the freezing process causes solutes and suspended particles to be concentrated in the unfrozen fraction[50]. At equilibrium, the composition of the unfrozen fraction must have a freezing point below the sample temperature. As cooling progresses to lower temperatures, the unfrozen solution will either crystallize or form an amorphous glass at a composition-dependent temperature (T_g'). Each of the sugars shown to be effective at preserving non-viral vectors readily form glasses during freezing[35,41]. In fact, Cherng and colleagues[51] explicitly state that they were careful to use freezing conditions under which the excipient forms a glassy state. Thus, it could be argued that the observed protection derives from the immobilization of vectors within a glassy matrix that prevents aggregation.

This effect could also explain the observation that complexes frozen rapidly to –70°C and stored at –20°C maintained transfection rates, whereas samples frozen directly to –20°C were damaged[44]. Under these experimental conditions, the rapid freezing to –70°C probably caused the formation of a protective sorbitol glass ($T_g' = -43$°C) that would not have formed in samples frozen directly to –20°C. Although storage at –20°C would not have maintained sorbitol in the glassy state indefinitely, crystallization of excipients under these conditions could take years and so preservation on a relatively short timescale might be achieved. However, prolonged stability under these conditions can be extremely sensitive to brief exposures to elevated subzero temperatures like those found during defrost cycles in a conventional freezer.

Several studies have shown that cryoprotection depends on the concentration of stabilizing excipient[31,43]. Recent studies have further demonstrated that the excipient concentration that is sufficient to protect complexes during freezing depends on the particle concentration: more dilute suspensions require less excipient for protection[43]. As glass formation during freezing is independent of the initial excipient concentration, these findings suggest that mechanisms other than glass formation contribute to cryoprotection. Furthermore, our work has clearly shown that some glass-forming excipients (e.g. hydroxyethyl starch) do not preserve particle size during freezing[43]. These findings strongly suggest that stabilization during freezing cannot be solely because of glass formation.

Particle isolation

As described above, solutes and suspended particles are concentrated in the unfrozen fraction during the freezing process. The volume of the unfrozen fraction at any temperature is determined by the initial solute concentration[50]. Therefore, the observation that more dilute suspensions require lower initial excipient concentrations for cryoprotection suggests that 'crowding' of particles facilitates aggregation[43]. In fact, Anchordoquy and coworkers[43] recently demonstrated that there is a crucial excipient:DNA ratio at which protection is observed. Furthermore, they concluded that the volume of the unfrozen fraction at this ratio is sufficient to isolate DMRIE (1,2-dimyristoyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide)–cholesterol–DNA (DMRIE–C–DNA) complexes in a viscous excipient matrix, thereby preventing aggregation[43]. An excipient:DNA ratio of approximately 1000 (by weight) was sufficient to achieve complete protection by either sucrose or glucose, but only partial protection was observed in solutes that crystallize during freezing, such as mannitol[43].

These findings are consistent with studies from other laboratories, and suggest that results should be reported in

Table 1. Freeze–thawing studies of DNA-based therapeutics

Cationic agent	Formulation	Freezing protocol	Storage
DOSPA/DOPE (Ref. 21)	5% Dextrose	Frozen at –20°C	3 months
PDMAEMA (Refs 51,54)	10% Sucrose, trehalose, lactose	Frozen on precooled (–38°C) shelf	–
DOTAP/cholesterol/protamine[29]	5% Sucrose	Frozen at –20°C (30 min) then –80°C; Frozen directly at –80°C; liquid nitrogen	–
DMRIE/cholesterol[31] DOTAP/DOPE DOSPA/DOPE	0.5 M Sucrose (~17%)	Frozen at –20°C; liquid nitrogen	–
DMRIE/DOPE (Ref. 44)	5% Sorbitol + 20 mM Sodium acetate	Frozen at –70°C, stored at –20°C	1 year
DMRIE/cholesterol[42]	1% and 5% Sucrose, trehalose, lactose, glucose, PEG, hydroxyethyl starch	Frozen to –38°C at –2.5°C min^{-1}	16 hours
DMRIE/cholesterol[43]	Sucrose or glucose at 1000:1 sugar:DNA wt. ratio + 2.5 mM Tris-HCl	Frozen to –38°C at –2.5°C min^{-1}	16 hours

Abbreviations: DOSPA, 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium; DOPE, dioleoylphosphatidyl ethanolamine; PDMAEMA, poly(2-dimethylamino)ethyl methacrylate; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMRIE, 1,2-dimyristoyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide.

terms of excipient:DNA-weight ratio rather than excipient concentration. More recent studies have shown that an excipient:DNA ratio of 1000 can protect other lipid–DNA complexes but that polymer–DNA complexes require much greater ratios to prevent aggregation during freezing[52]. In addition, we have observed that simply preventing aggregation is insufficient to maintain transfection rates of certain non-viral vectors during freezing. This suggests that interactions between cationic agent and DNA within a complex are crucial for gene delivery, and that they can be perturbed by the freeze–thawing process. Clearly, more work is needed to characterize and understand these subtle interactions at the molecular level.

Regardless of the mechanisms by which excipients might protect non-viral vectors during freezing, the evidence so far suggests that frozen formulations might offer pharmaceutically relevant stability to some types of complexes (Table 1). However, the stability of frozen formulations can require strict maintenance of storage temperature to prevent thawing and/or crystallization of excipients. Maintaining these conditions during transportation is difficult, especially if worldwide distribution is desired. Furthermore, the extra costs associated with the shipping and storage of frozen formulations can be substantial, even within the USA. These problems have generated interest in developing dehydrated formulations that are stable at ambient temperatures.

Dehydrated formulations

Dried preparations offer the potential for prolonged stability at room temperature and can be ready to use after a simple rehydration step. In addition, rates of gene transfer to the lung have been shown to be increased by administering a dried formulation[53]. Although there are several methods of removing water from liquid formulations, the spraying process involves high shear forces that can damage non-viral vectors[40]. Therefore, spray drying of non-viral vectors has been avoided and stabilization studies have used freeze drying to generate dehydrated formulations.

The lyophilization process involves two stresses that are known to damage macromolecules: freezing and drying. As discussed above, slow cooling causes much more damage to complexes than rapid freezing[31,44]. Unfortunately, rapid cooling rates are not possible in contemporary lyophilizers, but many studies have attempted to hasten freezing by loading filled vials on precooled shelves[30,51,54,55]. Although this approach does speed cooling, the rates are still slow compared with those achieved with liquid nitrogen or by immersion in an ethanol–dry-ice bath[31,44]. As the latter approaches are not practical for large-scale production, we must apply the knowledge gleaned from the cryoprotection experiments discussed above to preserve vectors during the freezing step of the lyophilization process. However, it is crucial that freezing studies mimic the cooling rates and incubation times encountered in a freeze

Table 2. Lyophilization of non-viral vectors

Delivery vehicle	Formulation	Freezing protocol	Storage
DOTAP/DOPE (Ref. 57) DMRIE/Cholesterol	0.5 M Trehalose, sucrose, glucose	Liquid nitrogen	–
Transferrin/PEI (Ref. 55) Transferrin/polylysine/adenovirus adenovirus	10% Sucrose in HBS	Precooled shelf to –40°C	3 weeks
PDMAEMA (Refs 51,54)	10% Sucrose, trehalose, maltose	Precooled shelf to –40°C	–
PDMAEMA (Ref. 30)	10% Sucrose + 20 mM Hepes	Precooled shelf to –40°C	10 months
DOTAP/cholesterol/protamine[29]	10% Glucose, galactose, mannose lactose, maltose, sucrose, trehalose 1%, 5% glucose, lactose, trehalose	–20°C (30 min) then –80 (30 min); –80°C (30 min); liquid nitrogen	8 weeks
DMRIE/cholesterol[42]	5% Sucrose, mannitol, PEG, hydroxyethyl starch	–38°C at –2.5°C/min	–
Alkylated Cys-Typ-Lys_{18} (Ref. 38)	5% Sucrose, PEGylation	Dry ice	–

drying cycle if they are to be applicable to stability during lyophilization[43]. To this end, Anchordoquy and colleagues[42,43] used a freeze dryer and typical lyophilization protocol to conduct the insightful cryoprotection studies described above.

Although nature has perfected methods of protecting extremely complicated systems (even whole organisms) during freezing and drying[56], the prospect of stabilizing multicomponent assemblies (e.g. non-viral vectors) is a daunting challenge to a pharmaceutical scientist. Few things are more humbling than spending years trying to stabilize lipid–DNA complexes under carefully controlled conditions while realizing that a lowly bacterium routinely preserves not only its membranes and DNA but also its entire host of metabolic machinery during chaotic bouts with freezing and drying. A review of the literature reveals that lyophilization studies to date have focused on relatively simple systems such as purified proteins and synthetic lipid vesicles. The fact that multicomponent systems are extremely difficult to stabilize should be recognized by scientists who advocate the use of targeting ligands, endosmolytic peptides, nuclear localization peptides and condensing polymers in the development of non-viral vectors. However, it is encouraging that lyophilization studies to date have reported acute stabilization of polymer-based, lipid-based and adenoviral vectors (Table 2).

Similar to the stabilization achieved in freeze–thaw studies, protection during lyophilization has been reported when sugars are included in the formulation[29,30,42,51,54,55,57]. It is clear that the choice of excipient is crucial for protection during both freezing and drying[42,43]. However, the dilution of vectors in concentrated excipient solutions is problematic because gene delivery is most effective at high vector concentration[22,44]. Although rehydration with reduced quantities of water can effectively reconcentrate the formulation, it is usually required that reconstituted preparations do not greatly exceed isotonicity (e.g. 5% glucose, 10% sucrose).

High excipient concentrations can also make it necessary to extend the lyophilization cycle, which can significantly increase production costs[58]. It is also worth pointing out that even the cleanest sources of sugars contain trace metal contaminants that facilitate DNA degradation[36,37]. Unlike protein-based pharmaceuticals, chemical degradation of DNA can result in mutation as well as inactivation[59,60] and so the consequences of introducing altered polynucleotides that act as a template for mRNA, and ultimately protein production, could be substantial. For example, oxidized deoxyguanosine can pair with adenosine instead of cytosine[59,60]; if this occurs during transcription, the mutated mRNA would be translated into a mutated protein *in vivo*, potentially triggering a life-threatening immune reaction. This potential problem is not generally appreciated and warrants investigations into the chemical stability of DNA-based therapeutics on pharmaceutically relevant timescales (>2 years).

The mechanism by which excipients stabilize non-viral vectors during lyophilization has yet to be elucidated. In addition to stresses encountered during freezing, dehydration is known to perturb macromolecular integrity[42]. In the case of aqueous formulations of non-viral vectors, the removal of water eliminates the suspending medium and

creates contact between particles in the dried state. The incorporation of excipients before lyophilization would allow particles to be separated in a matrix that might be maintained in the dried state if collapse was avoided[58,61]. This situation would minimize interactions between particles and inhibit aggregation. Considering that sugars are known to form amorphous matrices during dehydration, maintenance of particle size during lyophilization might simply be because of glass formation, assuming that no damage occurred during freezing. This is consistent with a recent report by Kwok and coworkers showing that non-viral vectors can be stabilized without sugars during drying by incorporating PEGylated components into the particle[38]. However, similar experiments with PEGylated lipid–DNA complexes have clearly shown that formulation with stabilizing excipients is still necessary (L. Girouard, unpublished observation). Although the recovery of particle size has been correlated with transfection rates, recent work has demonstrated that gene delivery can be impaired despite maintenance of particle size[52]. Therefore, as discussed above, it appears that interactions between the cationic agent and DNA within a complex can be disrupted without altering particle dimensions. Unfortunately, there is little information about what type of interactions are needed for successful gene delivery, but it is not surprising that physical stresses like dehydration can disrupt crucial structural characteristics.

Although entrapment in a glassy matrix alone is unable to preserve macromolecular structure, sugars have also been shown to preserve the integrity of dried liposomes and proteins by forming hydrogen bonds with the macromolecule, thereby mimicking the hydrated condition[62–64]. This concept has been termed the 'water replacement hypothesis' by Crowe and colleagues[63,64], and has been implicated in the preservation of non-viral vectors in the dried state[29,42,58]. However, recent studies have clearly documented that lyophilization in the presence of sugars does not completely preserve transfection activity during dehydration[42]. Considering the unique structures that have been suggested for some non-viral vectors[65], water replacement via hydrogen bond formation might not be enough to stabilize these novel pharmaceuticals. More work is needed to understand the interactions between excipients and non-viral vectors so that lyophilized formulations with prolonged stability can be developed.

Conclusions

Aqueous formulations of non-viral vectors are unstable and it is doubtful whether sufficient stability can be attained in liquid suspensions. Studies have shown that frozen formulations can offer prolonged stability in some cases, but concerns associated with the potential for thawing and the costs of maintaining the frozen state during shipping and storage have generated interest in developing dehydrated formulations.

To date, lyophilization studies have reported that some non-viral vectors can be stabilized during acute freeze–drying stress, but prolonged storage studies are lacking. Unfortunately, most studies in this area have taken an empirical approach and provide little insight into the physical mechanism(s) by which excipients stabilize DNA-based therapeutics. In addition, there is clearly a need to characterize the interactions between cationic agents and DNA more clearly within the non-viral vector, to understand the nature of the nanoscopic structures that must be preserved to retain activity.

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