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PRACTICE AND TECHNOLOGY OF CELLULAR DRUG DELIVERY

Formulation Considerations for DNA-Based Therapeutics

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1. Introduction

The ability to deliver genes to cells and tissues *in vivo* offers the potential to develop potent vaccines and treat many hereditary diseases that are currently considered incurable, e.g., cancer, cystic fibrosis (CF), severe combined immunodeficiency (SCID), and acquired immune deficiency syndrome (AIDS) (1–6). Considering the tremendous promise of DNA-delivery technology, in addition to the extensive genetic information now available from the Human Genome Project, it is not surprising that gene therapy is being touted as the next revolution in medicine. Although a strict definition of “gene therapy” would be limited to therapeutic approaches that aim to use polynucleotides as a template for the *in vivo* production of proteins, the term is often used to refer to a wide variety of strategies that employ nucleotide-based molecules (e.g., vaccines, antisense, ribozymes, siRNA). To date, 70 clinical protocols have been approved for the delivery of naked DNA, comprising approx 11% of the total number of gene-therapy clinical protocols (<http://www.wiley.co.uk/genetherapy/clinical>). Slightly more studies (~12%) have employed nonviral, lipid-based vectors to facilitate DNA delivery. In comparison, the large majority of clinical gene therapy trials (>70%) utilizes viruses to deliver therapeutic genes because viruses are more efficient than contemporary synthetic gene-delivery systems. The higher efficiency of viruses should not be surprising if we recognize that these organisms have been evolving their gene-delivery machinery for billions of years. In contrast, the development of nonviral systems for therapeutic gene delivery can be traced back a mere 15 years (7). Although more efficient nonviral gene-delivery systems continue to be developed, synthetic systems have yet to replicate the efficiency of viruses. One significant drawback of viral delivery is the immunogenicity of viruses, which causes significant inflammation *in vivo* (8), and eliminates the potential for multiple dosing (anyone who has ever had a cold is familiar with the fever and inflammation associated with an immune response to viruses). In fact, the adverse reactions associated with viral delivery have been implicated as the cause of death in clinical trials (9,10). Also, a clinical trial involving liver infusion of an adeno-associated virus (AAV) for the treatment of hemophilia B was halted because of the presence of the viral vector in patient semen, and the concern that the genetic alteration could be passed to offspring (11). More recently, two patients treated with *ex vivo* gene

therapy for the treatment of SCID have developed leukemia owing to insertional mutagenesis caused by the retroviral vector used in the study (12,13). Considering the potential safety risk involved in employing viruses as a therapeutic moiety, there is renewed interest in developing safe, efficient, nonviral gene-delivery systems.

Recent studies have reported that synthetic vectors transfer genes at rates only three- to fourfold lower than adenoviruses, and that the overall efficacy of nonviral vectors is actually greater because of the high cytotoxicity and immunogenicity of the adenovirus (14). In addition to the obvious safety advantages of avoiding viral vectors, the lack of a specific immune response to synthetic systems allows them to be readministered multiple times to increase transfection rates and prolong therapeutic gene expression (15–18). Despite these advantages, nonviral 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 nonviral gene-therapy research on improving transfection efficiency. It is intriguing that recent studies have suggested that delivery may not be as much of a barrier as is typically assumed, and that epigenetic events pertaining to the expression of the therapeutic gene deserve increased attention (19–21). Furthermore, research on the plasmid DNA-mediated, specific, cytotoxic T-cell response to tumors (22,23) and small, interfering, double-stranded RNAs (24,25) have identified therapeutic approaches that may not require the high-transfection efficiencies necessary in traditional gene therapy. Although these developments represent promising avenues for nucleotide-based therapeutics, critical pharmaceutical problems (e.g., physicochemical instability of DNA-based therapeutics) will need to be resolved before these technologies are widely deployed. Unfortunately, the study of these less glamorous aspects of gene therapy has received little attention.

It is important to recognize that the widespread use of nucleotide-based therapeutics will require the bulk manufacturing of polynucleotides. For some purposes (e.g., antisense, siRNA, ribozymes), the relatively short strands (≤ 50 bases) of oligonucleotides can be chemically synthesized, and companies that specialize in the production of oligonucleotides are capable of producing tons of purified material. In contrast, larger sequences (typically > 3000 base pairs) encoding genes are synthesized in bacteria as double-stranded, supercoiled plasmids. Extensive purification procedures are subsequently employed to ensure that the isolated product contains the highest possible percentage of plasmids in the supercoiled form. A single cleavage event in the backbone of either polynucleotide chain causes the release of torsional energy in the supercoils, and the conversion to the open circle form (26). Although backbone cleavage can result from any one of a variety of chemical mechanisms (26–29), retention of the supercoiled form serves as an indicator of chemically stable conditions. In practice, industry strives to maintain the highest possible supercoil content ($\geq 90\%$; 30). In contrast to the enormous quantities previously mentioned for oligonucleotides, the production of *gram* quantities of purified supercoiled plasmid is usually sufficient for Phase I clinical trials for most applications.

As described earlier, the maintenance of supercoil content is used routinely as the primary measure of the chemical integrity/stability of naked DNA, and is routinely assessed with agarose gel electrophoresis. The stability of nonviral gene-delivery systems (i.e., DNA bound to agents that facilitate delivery) is more complicated, and involves aggregation of suspended particles and the retention of poorly characterized structural motifs, in addition to the maintenance of supercoil content. Considering the significantly different obstacles and corresponding strategies involved in the stabiliza-

tion of a solution of naked DNA vs a suspension of DNA-containing particles, this chapter will address these challenges separately. In addition to discussing liquid formulations, we describe progress to date in the development of frozen and dehydrated preparations of both naked DNA and nonviral gene-delivery systems. Wherever possible, we attempt to provide some mechanistic insight into the findings described in the literature with the hope that future formulations may be rationally designed rather than empirically derived.

2. Naked DNA

Many cellular targets of gene therapy involve the use of delivery vehicles (e.g., viruses, cationic liposomes); however, Wolff et al. (31) have demonstrated that naked DNA is taken up readily by muscle cells *in vivo*. This observation has led to the development of DNA vaccines that are known to elicit both a humoral and cellular immune response (4–6,32). These potent vaccines are currently being tested in Phase I/II clinical trials, and are being combined with traditional protein vaccines and/or adjuvants (e.g., interleukin-2 [IL-2]) to further boost immune responses to pathogenic parasites, viruses, bacteria, and cancer. With all of the promise surrounding nucleic acid-based therapeutics, surprisingly little attention has been paid to the chemical stability of DNA on a pharmaceutically relevant time-scale. Clearly, marketable nucleotide-based products (e.g., vaccines) will need to possess extended shelf-lives similar to other biopharmaceuticals (18 mo to 2 yr).

DNA, like other biopharmaceuticals (e.g., proteins, liposomes), is susceptible to many degradation reactions that are enhanced in aqueous formulations. However, unlike other biopharmaceuticals, the DNA used in gene therapy is utilized as a template for protein synthesis within the patient. It is imperative that we recognize this distinct difference between gene therapy and the administration of other biopharmaceuticals because chemical alterations of the nucleotide bases has been shown to result in mispairing that can ultimately lead to the intracellular production of mutated proteins (33–35). Considering that mutations that result in altered protein folding can be recognized as foreign by the host's immune system, chemical alteration of DNA has the potential to trigger a life-threatening immune response to an endogeneously synthesized protein encoded by the "therapeutic" plasmid. Although the probability of this event is quite low, it behooves companies that are developing DNA-based therapeutics to utilize rigorous standards for maintaining chemical integrity.

To avoid chemical and enzymatic degradation in a laboratory setting, researchers commonly store DNA as a precipitate in ethanol at -80°C . Under these conditions, nucleic acids are stable for prolonged periods, but must be isolated from the ethanol, transferred to aqueous buffers, and quantified prior to use. These manipulations preclude ethanol precipitates from being used for clinical DNA preparations. Instead, it would be ideal if aqueous solutions could be formulated to resist depurination (36), depyrimidination (37), deamination (38), and hydrolytic cleavage (39,40). To this end, studies have attempted to formulate in alkaline conditions to inhibit these acid-catalyzed degradation mechanisms during prolonged storage (26,27). Under such conditions (pH 8.5), the primary threat to DNA stability (excluding nuclease contamination) is oxidation (26,27). (It is worth noting that lipids used in gene-delivery systems are rapidly degraded under these conditions [41]; a serious dilemma for companies developing lipoplexes as pharmaceutical products.) In general, oxidative damage is greatly enhanced by the presence of trace metals (e.g., Fe^{3+} , Cu^{2+}) that produce hydroxyl radi-

cals via Fenton-type reactions (26–29). In their classic study, Evans et al. demonstrated that transition metal impurities in various formulation components (including the purified DNA preparation) permitted significant levels of oxidation to occur during storage (27). The rate of oxidation was attenuated when buffers were demetalated prior to storage. Although these findings demonstrate that trace-metal contaminants are responsible for the observed oxidation, the procedures used to remove trace metals for a small laboratory experiment are not practical for large-scale purification and production (27,42). Therefore, chelators and antioxidants will need to be included in DNA formulations to reduce oxidation and maximize stability during storage. To this end, Evans et al. demonstrated that the combination of 200 μM ethylenediaminetetraacetic acid (EDTA) and 1% ethanol is capable of preserving more than 90% of the initial supercoil content during 2 yr of storage at room temperature (27). It is worth noting that EDTA does not bind metals in such a way that prevents hydroxyl radical formation and subsequent oxidation (43), but the presence of ethanol as a radical scavenger is sufficient for prolonged storage in the liquid state. However, the use of ethanol is not compatible with some adjuvants and delivery systems that may be desirable in a DNA vaccine formulation (27). For example, the presence of ethanol would solubilize lipids incorporated into the formulation. Therefore, it would be advantageous to develop formulation strategies that eliminate the need for ethanol. As shown by Evans et al., the use of 200 μM diethylenetriaminepentaacetic acid (DTPA) enhances stability to an extent comparable to that observed with EDTA/ethanol (27). These findings suggest that the employment of DTPA might circumvent the need for ethanol, thereby achieving stable liquid formulations that are more widely compatible with pharmaceutical applications.

To test the ability of DTPA to preserve DNA integrity during long-term storage, we monitored the room temperature stability of aqueous DNA formulations (50 $\mu\text{g/mL}$, 2.5 mM Tris-HCl, pH = 8.5) with and without 200 μM DTPA (Fig. 1). The data indicate that the presence of DTPA significantly enhances the preservation of supercoil content during prolonged storage at room temperature. In addition, Fig. 2 shows that the preservation of biological activity of the plasmid correlates with the observed maintenance of supercoil content. However, the loss of supercoil content and transfection rates are much too rapid under these conditions, suggesting that aqueous formulations lacking ethanol do not provide sufficient stability during prolonged storage at room temperature. It is important to note that DNA stability can be a limiting factor even in instances where DNA is incorporated into a delivery system, and therefore the problems associated with chemical stability are not limited to naked DNA formulations. Although other chelators (e.g., desferal, inositol hexaphosphate) and scavengers (e.g., ascorbic acid, trolox, cysteine) might prove more effective and thereby eliminate the need for ethanol, sufficient stability on a pharmaceutical time scale may require the development of frozen or dehydrated formulations.

The effect of freeze-thawing on DNA was first reported by Shikama more than 35 yr ago (44). This study demonstrated that the double-helix of calf thymus DNA remained intact during freezing to temperatures as low as -192°C (44). Several years later, Lyscov and Moshkovsky described a mechanism of DNA degradation during freezing (“cryolysis”), that was dependent on the rate of cooling of the frozen sample (45). These authors suggested that cryolysis resulted from the formation of cracks within the ice during freezing, and demonstrated that these cracks were more prevalent in rapidly cooled samples (45). In contrast, Davis et al. showed that slow freezing is more damag-

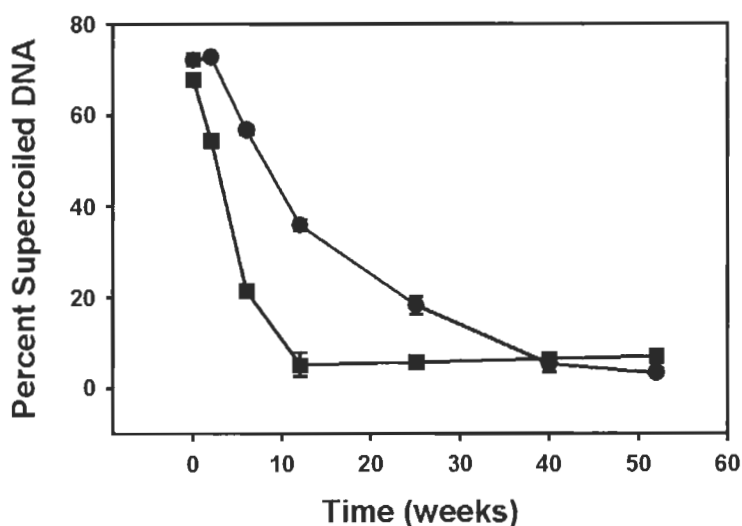


Fig. 1. Supercoil content of naked DNA after prolonged storage in an aqueous formulation. Plasmid DNA (50 μ g/mL) was formulated in 2.5 mM Tris-HCl, pH 8.5, either with (circles) or without (squares) 200 μ M DTPA, and stored at room temperature for 1 yr. Supercoil content was assessed with agarose gels followed by ethidium bromide staining and quantification. Each symbol represents the mean \pm 1 standard error of single measurements on triplicate samples.

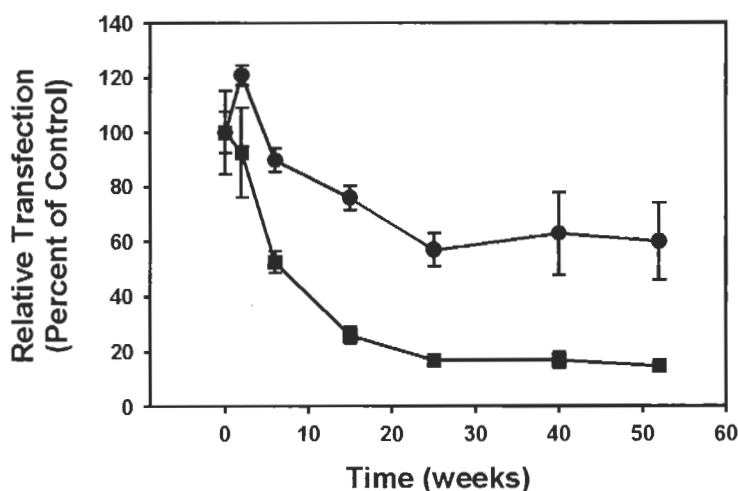


Fig. 2. Transfection rates of naked DNA after prolonged storage in an aqueous formulation. Plasmid DNA (50 μ g/mL) encoding green fluorescent protein was formulated in 2.5 mM Tris-HCl, pH 8.5, either with (circles) or without (squares) 200 μ M DTPA, and stored at room temperature for 1 yr. At the indicated time-points, aliquots were removed and complexed with polyethylenimine (N/P = 7). The resulting complexes were used to transfect COS-7 cells in culture, and the percentage of cells expressing the encoded gene was quantified by FACS. Transfection rates are expressed as a percent of that for complexes prepared with fresh DNA (typically 40–60% of cells expressing). Each symbol represents the mean \pm 1 standard error of single measurements on triplicate samples.

ing than rapid freezing, and that damage to oligonucleotides is dependent on base composition, structure, and length (46). In a study by Ando et al., very rapid cooling by immersion of samples into liquid nitrogen was used to protect DNA from shear stress during homogenization (47). However, these authors clearly demonstrated that both sugars and EDTA were required to preserve supercoil content during freezing (47). Although it is not clear whether these excipients specifically protect against the cryolysis mechanism of degradation described by Lyscov and Moshkovsky, it seems possible that glass formation, caused by the presence of sugars (48), would attenuate the cracking that is implicated in DNA degradation. As suggested by the authors, the presence of EDTA might inhibit DNase (47). Additionally, EDTA could serve to chelate transition metals that are known to facilitate DNA degradation (27). Surprisingly, we could not find a single report in the literature that investigated the storage stability of naked DNA in a frozen formulation. However, distribution of a frozen formulation is complicated by the potential for thawing, and the need for a dependable cold chain. It would therefore be advantageous to develop dehydrated preparations that could be shipped and stored at ambient temperatures.

With regards to the stability of DNA during dehydration, it is quite common for molecular biologists to dry down aqueous or ethanolic solutions of DNA for use in their experiments. Because dried samples are not stored for prolonged periods in the dried state, and because molecular biological techniques are based on gene amplification and precise selection of the desired products among large numbers of imperfect reaction products, DNA damage is not detected. Unfortunately, this situation leads to the common misconception that DNA is a stable molecule. This naïveté on the part of molecular biologists permeates the field of gene therapy, and is largely responsible for the lack of studies investigating stability in a pharmaceutical context. Obviously, the sequence fidelity necessary to satisfy the experimental conditions in a typical molecular biology laboratory is drastically different than that required for a federally regulated, pharmaceutical production facility.

There are several methods of removing water from liquid preparations to produce dehydrated DNA formulations, e.g., spray-drying, spray freeze-drying, lyophilization. Although the small plasmids currently employed in gene therapy (~5 kb) are quite resistant to shear stress, spraying of DNA has largely been avoided owing to the common misperception that the shear-induced damage reported in early studies on genomic DNA is generally applicable to other forms, e.g., plasmids and oligonucleotides (49–52). As a result, the majority of stabilization studies have employed freeze-drying (lyophilization) to generate dehydrated formulations. With the notable exception of lyophilization studies by Poxon and Hughes (53) and Pikal et al. (54), we find it surprising that so little attention has been paid to the lyophilization of naked DNA (53,54). Incidental reports available in the literature are contradictory; some suggest that dehydration might be stabilizing (55,56), whereas others show that lyophilization induces damage (47,57–59). Recent studies have demonstrated that sugars can protect DNA against damage (e.g., loss of supercoil) induced during lyophilization (47,53). However, storage-stability studies on lyophilized DNA are lacking. In their study of lyophilized DNA, Poxon and Hughes (53) reported that lactose, glucose, and sucrose were able to preserve the biological activity of naked DNA stored at 75°C for 3 wk (53). With the exception of that brief storage study, the only study to investigate the stability of naked DNA during prolonged storage in the dried state concluded that degradation

occurs gradually over 14 mo at 4°C (58). Considering that this study was conducted almost 30 yr ago, before the advent of modern molecular biology, there is an obvious need for extended storage studies on naked DNA dried in stabilizing sugar formulations.

It has been reported that lyophilization causes some denaturation of double-stranded DNA (53). This finding is consistent with earlier work by Lindahl, who observed that DNA stored over phosphorous pentoxide does not retain its double-helical structure, which renders it more vulnerable to oxidative damage during storage (60). It is interesting that spores that have evolved to survive prolonged periods in a quiescent state synthesize proteins that bind to the dehydrated A conformation, reducing rates of depurination by at least 20-fold (60). Bacteria also respond to environmental stress by synthesizing proteins that co-crystallize with DNA, such that oxidation is greatly reduced (61). A similar role has been suggested for chromatin in mammalian cells (62). It follows that it may be possible to compact DNA such that bases become sequestered and less susceptible to reactive oxygen species (ROS) during prolonged storage. This suggestion is analogous to the “base-shielding hypothesis” that was first proposed by Ward and Kuo (1978) to explain the greater resistance of duplex DNA (63), as compared with single-stranded polynucleotides, to chemical degradation (37,64,65). Thus, the compaction of DNA may serve to enhance stability, but the use of cationic agents that bind to DNA is also used to facilitate delivery. Because DNA bound to compacting molecules technically is not “naked,” it seems appropriate to consider the stability of DNA complexed with cationic agents in the following section on nonviral gene-delivery systems.

3. Nonviral Gene-Delivery Systems

Although improved nonviral vectors are continually being developed, it is generally recognized that the stability of these systems is insufficient for widespread application of this promising technology (66–68). Therefore, it would be advantageous to develop methods of stabilizing nonviral vectors that allow preparations to be produced under optimal conditions, shipped, and stored until needed. Considering the massive research effort focused on improving the delivery efficiency of nonviral vectors, potent DNA-based therapeutics for the treatment of many diseases may soon become a reality. However, if synthetic delivery vehicles cannot be rendered sufficiently stable to be developed as pharmaceutical products, it is unlikely that these therapies will ever be employed to treat disease. Therefore, the realization of the enormous potential of nonviral gene therapy will ultimately require studies that investigate vector stability and characterization. Such studies will need to be conducted so that vectors with consistent physical and chemical properties can be manufactured on an industrial scale. Currently, nonviral vectors are made in relatively small batches by mixing a solution of DNA with a solution/suspension of an agent that facilitates delivery (e.g., lipid, polymer). Although there have been some interesting reports of neutral polymers enhancing transfection rates despite minimal interaction with the plasmid (69,70), most delivery systems incorporate cationic agents that interact electrostatically with DNA to form complexes that are ultimately used for gene delivery (71). This method of preparation typically results in a highly heterogeneous suspension of particles possessing a wide range of \pm charge ratios and size (72–76). Furthermore, the particle size, zeta potential, and other biophysical characteristics that are known to affect gene-delivery

efficiency can be altered by many factors including mixing protocol, buffer components, and complexation time (77–81). These issues complicate scale-up and manufacturing, and will clearly need to be addressed if nonviral gene-delivery proves clinically efficacious.

Assuming that adequate industrial-scale manufacturing procedures can be developed, it is imperative that the biophysical properties and gene-delivery efficiency of nonviral vectors be preserved until the product is administered in the clinic. It is important to emphasize that the biophysical properties (e.g., size) must be maintained in order to insure appropriate quality control of a pharmaceutical product, regardless of the ultimate effects on gene delivery. To this end, stability becomes an issue for the relatively small-scale preparations currently being used for Phase I trials, even though such studies are not designed to test therapeutic efficacy. Clearly, instability of the preparation after production will adversely affect quality control, safety, and transfection rates. 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), our discussion focuses on the physical stability of nonviral vectors, and the maintenance of particle characteristics during processing and storage.

It is well-known that aqueous suspensions of nonviral vectors have a tendency to aggregate over time (67,73,82). The combination of multiply charged species in this manner results in incomplete charge neutralization, and a heterogeneous suspension of vectors possessing a range of net charge and particle sizes (73). It is this heterogeneity combined with unneutralized regions within complexes that fosters aggregation in aqueous suspensions (83,84). The time-scale over which aggregation occurs is typically from minutes to hours, which is clearly insufficient for a pharmaceutical product (68,85). Several reports claim to have developed “stable” nonviral vectors that do not aggregate for several hours (17,86–88). Although this level of stability may be adequate for some experimental purposes, such short half-lives are clearly insufficient for widespread clinical use. Some clinical trials have attempted to circumvent this problem by preparing complexes at the bedside, immediately prior to injection (15,89). This method of sample preparation and administration is not practical, and leads to significant variations in product quality, safety, and gene-delivery efficiency. This problem has stimulated an interest in developing stable, single-vial formulations that resist aggregation and possess consistent physical properties.

Studies have employed different approaches in an attempt to prevent aggregation of pre-assembled nonviral vectors. For example, Caplen et al. diluted complexes with an alkaline solution that was intended to enhance stability by weakening the interaction between the cationic lipid and DNA (90,91). Using another approach, Hofland et al. and Gao and Huang employed sucrose density gradients to separate the heterogeneous population of particles, and isolate a stable fraction of lipid/DNA complexes (92,93). These stable fractions could be stored under refrigerated conditions for 3 mo without detectable losses in transfection rates. It was subsequently demonstrated that these complexes effectively deliver genes *in vivo* (94). 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 earlier indicate that lipid/DNA complexes are more stable if they are separated from other components in the bulk suspension. Considering that preparations of lipid/DNA complexes typically contain free (i.e., uncomplexed) liposomes and/or

naked DNA, these findings suggest that interactions between complexes and free components can result in decreased rates of gene delivery. Furthermore, it has been reported that aggregated nonviral vectors are significantly more toxic, demonstrating that instability ultimately can lead to safety concerns (86). Such effects are not surprising if we consider the impact that incorporation of additional polynucleotides and/or fusion of excess cationic liposomes would have on a vector's charge, size, and lipid-DNA interactions. In order to enhance stability, it should be possible to develop methods of preventing interactions among components within a single suspension. In fact, Hong et al. and Eastman et al. utilized polyethylene glycol (PEG)-lipid conjugates to prevent aggregation of nonviral vectors and sterically stabilize particles in aqueous suspensions (95,96). Studies have also utilized PEG derivatized to anionic peptides in order to coat cationic particles and reduce aggregation with serum components (86,97,98). Although the use of PEGylated components appears to reduce aggregation of nonviral vectors under some conditions, PEGylated lipid/DNA complexes appear to be more sensitive to agitation stress than vectors lacking PEG (99). It has been suggested that the PEG chains extending from vector surfaces become entangled during agitation, thereby facilitating aggregation and/or fusion (99). In addition, steric stabilization by PEG is also known to curtail interactions with cells and interfere with cellular processing (75,100). Therefore, nonviral vectors containing PEGylated components typically display lower transfection activities than their non-PEGylated counterparts, despite improved stability in physiological solutions (99,100).

The use of condensing polyamines has also been shown to increase the stability of nonviral vectors, and prevent aggregation during storage (95,101). Although it is doubtful that stabilization via condensation would be sufficient for prolonged storage as an aqueous preparation, studies by Cherng et al. report that suspensions of poly(12-dimethylamino)ethyl methacrylate)-based gene-delivery systems maintain particle size and *in vitro* transfection rates for 10 mo at 20°C (102). This report of prolonged stability of an aqueous vector suspension is exceptional, and differs substantially from the short shelf lives (hours to days) that are typical of most synthetic gene-delivery systems. One factor that plays a significant role in determining stability in liquid suspensions is the concentration of both DNA and cationic agent(s) (103). As the suspension gets more concentrated, there is a higher probability for aggregation. It follows that very dilute suspensions that are suitable for *in vitro* transfection studies exhibit markedly improved stability in liquid formulations as compared to when the identical vector is prepared under the concentrated conditions necessary for *in vivo* delivery (103). In addition, it has been shown that prolonged storage causes lipid/DNA complexes to dissociate gradually over 70 d (104). Although the increased stability of some of the aqueous formulations described previously is encouraging, it should be noted that storage-stability studies typically neglect stresses that are commonly encountered during processing and/or shipping, e.g., agitation and/or freeze-thawing (99,105,106). Considering that both of these stresses can alter physical characteristics and significantly reduce transfection rates of nonviral vectors (67,68,105,106), it is prudent to test preparations under these conditions and develop formulations that can resist shipping-induced damage.

One method to circumvent the sensitivity of liquid formulations to shipping stresses would be to develop frozen formulations that are resistant to agitation-induced aggregation (Table 1). If we consider the intricate associations between cationic agent and DNA within a complex that are required for optimal gene delivery (e.g., structures

Table 1
Freeze-Thawing Studies of DNA-Based Therapeutics

Cationic agent	References	Formulation	Freezing protocol	Storage
DOSPA/DOPE	(92)	5% dextrose	Frozen and stored at -20°C	3 mo, transfection retained
PDMAEMA	(127,148)	10% sucrose, trehalose, lactose	Frozen on precooled (-38°C) shelf	—
DOTAP/cholesterol/protamine	(101)	5% sucrose	Frozen at -20°C (30 min) then -80°C ; Frozen directly at -80°C ; liquid nitrogen	—
DMRIE/cholesterol	(105)	0.5 M sucrose (17%)	Frozen at -20°C ; liquid nitrogen	—
DOTAP/DOPE				
DOSPA/DOPE				
DMRIE/DOPE	(103)	5% sorbitol + 20 mM sodium acetate 1% and 5% sucrose, trehalose, lactose, glucose, PEG, hydroxyethyl starch, Sucrose or glucose at 1000:1 sugar/DNA wt. ratio + 2.5 mM Tris-HCl	Frozen at -70°C , stored at -20°C	1 yr, transfection, lipid + DNA integrity retained
DMRIE/cholesterol	(107)		Frozen to -38°C at $-1^{\circ}\text{C}/\text{min}$	—
DMRIE/cholesterol	(108)		Frozen to -38°C at $-1^{\circ}\text{C}/\text{min}$	—
DOTAP/cholesterol	(109)	No excipient	Frozen in liquid nitrogen, stored at -80°C	20 d, zeta potential, size, transfection retained

involved in compaction, membrane fusion, complex dissociation), it is not surprising that freezing alters vector characteristics and performance (67,99,105). In addition to reductions in transfection activity, increases in particle size indicate that aggregation occurs during the freeze-thaw process (99,105,107,108). 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 (103,105,109). These observations suggest that the increased time during which particles can diffuse during slow cooling promotes aggregation of nonviral vectors, which results in reduced transfection rates (68,84,105,108). In fact, our experiments that assess particle size at different subzero temperatures after ice nucleation show that aggregation in the presence of excipients is attenuated at temperatures below -15°C (Fig. 3). Therefore, rapid freezing by immersion in liquid nitrogen presumably minimizes aggregation by reducing the time available for particles to collide in a liquid phase (103,105,109). Furthermore, the fact that maintenance of particle size often correlates with the recovery of transfection rates indicates that aggregation during freezing represents a major mechanism of damage. It is worth noting that an increase in particle size can result from irreversible phenomena (e.g., lipid fusion) or simple "clumping" (76). We have also observed that aggregation of some preparations is reversible (unpublished observations), but we have not determined whether dispersion of aggregates results in recovery of transfection activity. Because little attention has been paid to these different modes of "aggregation," further characterization of these different assemblies is clearly warranted.

The ability of "cryoprotectants" to prevent aggregation of proteins and liposomes during freezing is well-established (110–113). The manifestation of employing 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 dissolved in solution (e.g., proteins) as compared to suspended particles (e.g., liposomes). In the case of proteins, aggregation typically involves some "aggregation-competent" intermediate that is partially unfolded (114). Therefore, aggregation can be prevented by employing excipients to inhibit the formation of partially unfolded species (111,114). The mechanism by which stabilizing excipients, typically sugars, attenuate protein unfolding during freezing is the same mechanism that has been described by Timasheff and colleagues to explain solution stability, i.e., preferential exclusion (115,116). This thermodynamic mechanism involves the exclusion of solutes from the hydration shell of the protein, and the subsequent stabilization of the protein's native structure. For the purposes of this discussion, it is important to recognize that the preferential exclusion 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). In contrast, sugars are thought to stabilize liposomes by interacting directly with the membrane surface during freezing (110,112,113,117). The role of binding in membrane protection was unequivocally demonstrated by Hinch et al. who used galactose-specific lectins to protect thylakoid membranes during freeze-thawing (118). Although the implication of a direct interaction in stabilization does not preclude preferential exclusion of the excipient overall (i.e., a solute present at a high concentration on the membrane surface is still considered to be excluded if the bulk solute concentration is greater), it does suggest that the solute associated with the membrane surface is responsible for preserving vesicle integrity during freezing (110,112,113,117). Such an interaction between sugars and various membrane systems has also been observed in the solution state (119–122).

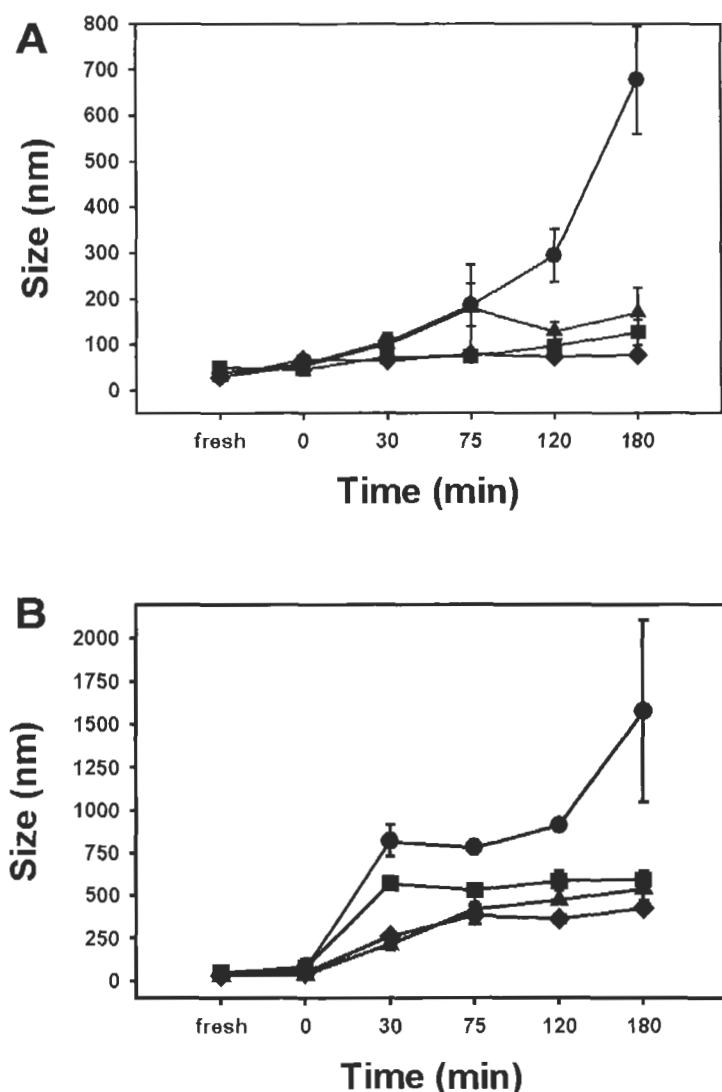


Fig. 3. Aggregation of polyethylenimine/DNA complexes after freezing. Vectors in either a sucrose (**A**; sugar/DNA = 200) or PEG (**B**; MW = 200 K, polymer/DNA = 125) formulation were cooled on a lyophilizer shelf that was ramped down to various temperatures at 2°C/min. When the sample temperatures reached approx -5°C, freezing was initiated by briefly dipping samples into liquid nitrogen such that ice was nucleated, but the sample was not extensively crystallized (time 0). Samples were then cooled on the lyophilizer shelf to the indicated sample temperatures, and held for 180 min. Under these conditions, sample temperatures reached -10°C (circles), -15°C (squares), -20°C (triangles), or -25°C (diamonds) by the 30-min time-point. Note that postnucleation sample temperatures of -10°C were insufficient to prevent aggregation. In contrast, vector size remained constant after achieving sample temperatures of $\leq -15^\circ\text{C}$ over the time course of this experiment. Each symbol represents the mean \pm 1 standard error of single measurements on triplicate samples.

In this way, solute-induced stabilization of suspended particles is analogous to the steric stabilization observed with PEGylated lipids (123,124).

The application of the stabilization mechanisms described earlier to the preservation of nonviral vectors has not been extensively studied. It is clear that there are many types of damage that can be incurred by nonviral vectors, e.g., chemical damage, aggregation, and structural alterations. Although all of these types of damage are important for quality control and transfection activity, stability studies typically only monitor changes in particle size and/or transfection as indicators of "recovery." Therefore, the available data limits the discussion of preservation mechanism to a deliberation of how solutes prevent vector aggregation during freezing. Nonviral vector preparations are similar to liposomes in that they are a suspension of particles that can be stabilized sterically by the incorporation of PEGylated components (86,95,97,98,124). Therefore, it seems plausible that the direct interaction mechanism described for liposomes (see earlier) might also apply to the stabilization of nonviral vectors. However, it should be pointed out that much greater amounts of sugar are required to prevent aggregation of nonviral vectors than that necessary to inhibit liposome fusion during freezing (110,125). The relatively large quantities of excipients suggest that maintenance of particle size is owing to nonspecific, "bulk" characteristics of the formulation. We have suggested two mechanisms that could potentially contribute to the observed retention of nonviral vector size during freezing: (1) glass formation and (2) particle isolation.

3.1. Glass Formation

The formation of ice during the freezing process causes solutes and suspended particles to be concentrated in the unfrozen fraction (126). At equilibrium, the composition of the unfrozen fraction must have a freezing point below that of 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 nonviral vectors readily form glasses during freezing (48,68). In fact, Cherng et al. explicitly state that they were careful to freeze under conditions where the excipient forms a glassy state (127). Thus, it could be argued that the observed maintenance of particle size derives from the immobilization of vectors within a glassy matrix that prevents aggregation.

Several studies have shown that maintenance of particle size is dependent on the concentration of stabilizing excipient (105,108). Recent studies have further demonstrated that the excipient concentration that is sufficient to retain vector size during freezing is dependent on the particle concentration, i.e., more dilute suspensions require less excipient to prevent aggregation (108). Because glass formation during freezing is independent of the initial excipient concentration, these findings suggest that mechanisms other than glass formation contribute to the maintenance of particle size.

Additional evidence against glass formation as the primary mechanism of stabilization comes from our work demonstrating that polymers that readily form glasses during freezing (e.g., hydroxyethyl starch) do not preserve particle size, whereas sugars (e.g., glucose) are able to maintain vector size at temperatures above T_g' (108). Although immobilization of suspended particles within a glassy polymer matrix is sufficient to prevent aggregation, many hydrophilic polymers possess high surface-tension increments that may foster coalescence in the unfrozen solution prior to vitrification. This effect can be significant considering that excipients are dramatically concentrated upon ice formation, with concentrations in the unfrozen solution typically reaching more than 60% by weight (108). The high concentration of excipients combined with

Table 2
Apparent Surface Tension of 5% PEG Solutions

Compound	MW	Surface tension	Standard error
Water	18	75.53	0.088
PEG	400	67.03	0.082
PEG	4600	64.03	0.216
PEG	20,000	63.93	0
PEG	200,000	60.36	0.525

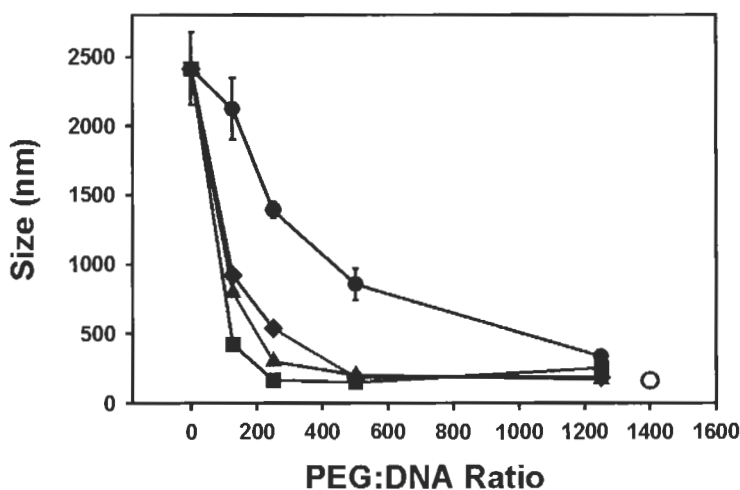


Fig. 4. Protection of DOTAP:DOPE/DNA complexes by PEG during freezing. Vectors were formulated in PEGs possessing molecular weights of 400 (circles), 4500 (triangles), 20 K (diamonds), and 200 K (squares). Samples were subjected to slow freezing ($\sim 2^{\circ}\text{C}/\text{min}$) to a shelf temperature of -40°C in a shelf lyophilizer. The shelf temperature was maintained overnight (~ 16 h), and vector size was monitored with dynamic light scattering after rapid thawing. The size of fresh vectors is indicated by the open circle at 1400 PEG/DNA ratio. Note that larger PEGs with lower surface tensions (Table 2) were progressively more effective at preserving particle size during freezing. Each symbol represents the mean ± 1 standard error of single measurements on triplicate samples.

subzero temperatures can result in surface tensions that are 30% higher than that observed in room-temperature solutions (108). According to this argument, polymers that possess negative surface-tension increments (e.g., PEG) should not foster coalescence during freezing, and thus might serve as superior glass-forming cryoprotectants. Although we have yet to thoroughly investigate this hypothesis, our results with different molecular-weight PEGs demonstrate that cryoprotection correlates with surface tension (Table 2, Fig. 4). However, these results are complicated by the fact that T_g' (48) and the affinity for the vector surface (128,129) varies with polymer molecular weight. Regardless, these findings strongly suggest that stabilization during freezing cannot solely be the result of glass formation. In addition, the data in Fig. 3 show that aggregation is attenuated by sucrose at temperatures well above its T_g' ($\sim 32^{\circ}\text{C}$). This suggests that the unfrozen solution at temperatures where particle size is maintained

(<-15°C) may be sufficiently viscous such that aggregation is inhibited on the experimental timescale (~16 h). Thus, glass formation, *per se*, may not be required under these conditions.

3.2. Particle Isolation

As described earlier, 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 (126). Therefore, the observation that more dilute suspensions require lower initial excipient concentrations for cryoprotection suggests that “crowding” of particles facilitates aggregation (108). In fact, Allison et al. demonstrated that there is a critical excipient:DNA ratio at which protection is observed (108). Furthermore, the authors concluded that the volume of the unfrozen fraction at this ratio is sufficient to isolate lipid/DNA complexes in a viscous excipient matrix, thereby preventing aggregation. An excipient/DNA weight ratio of approx 1000 was sufficient to achieve complete protection by either sucrose or glucose, but only partial protection was observed in solutes that crystallize during freezing, e.g., mannitol (108). These findings are consistent with reports from other laboratories, and suggest that results should be reported as a function of excipient/DNA ratio rather than the excipient concentration that is traditionally reported. Later studies reported that this excipient/DNA ratio can protect other lipid-DNA complexes and lipid-polymer-DNA complexes, but that polymer-DNA complexes require much greater ratios to prevent aggregation during freezing (125). It was subsequently determined that the purity of the polymer and DNA can dramatically affect stability during physical stress, and that cleaner preparations of polymer/DNA complexes are also stabilized during freezing at an excipient/DNA weight ratio of 1000 (unpublished results).

As noted earlier, it is critical for quality control and safety that vector size be maintained during processing regardless of its effect on transfection rates (99). As a result, size is typically monitored during stability studies. However, the ability of vectors to deliver genes (i.e., transfection rates) must also be preserved. Not surprisingly, we have demonstrated that simply preventing aggregation is not sufficient to maintain transfection rates of nonviral vectors during freezing (125). These latter findings suggest that critical interactions between cationic agent and DNA within a complex can be perturbed by the freeze-thawing process. Clearly, more work is needed to characterize and understand these subtle interactions at the molecular level. Fortunately, recent work with a variety of biophysical techniques has begun to characterize interactions within nonviral vectors (130–135), but it is still unclear which physical properties are critical for gene delivery.

Regardless of the mechanisms by which excipients might protect nonviral vectors during freezing, evidence to date suggests that frozen formulations may offer pharmaceutically relevant stability to some types of complexes (103). However, the stability of frozen formulations can require strict maintenance of storage temperature to prevent thawing and/or crystallization of excipients (68). It is important to note that prolonged stability in the frozen state can be extremely sensitive to very brief exposures to elevated subzero temperatures, e.g., during defrost cycles in a conventional freezer. Maintaining strict control of sample temperature during transportation is difficult, and can limit worldwide distribution, e.g., consider the shipment of a frozen formulation to the third world. Furthermore, the extra costs associated with the shipping and storage of frozen formulations can be substantial, even within the United States. These problems

have generated interest in developing dehydrated formulations that are stable at ambient temperatures.

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 administration of a dried formulation (136). Although there are several methods of removing water from liquid formulations, the spraying process involves high shear forces that can damage nonviral vectors (52,137,138). However, several studies have demonstrated that the complexation of DNA with cationic agents greatly stabilizes polynucleotides during spraying (139,140), but that cationic agents differ greatly in this regard (137,138). These findings suggest that specific interactions with the polynucleotide chain may 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.

Spray-drying of nonviral vectors is starting to receive more attention, and recent studies by Seville et al. suggest that spray-drying might offer significant advantages over traditional freeze-drying (141). Our preliminary experiments have investigated spray freeze-drying and shown that, although vector size is maintained in the presence of sugars, transfection rates are greatly reduced (unpublished observations). The majority of stabilization studies have employed freeze-drying to generate dehydrated formulations (Table 3). Curiously, numerous studies have reported increases in transfection activity after freezing and/or dehydration (84,101,107,141–143). This enigmatic effect typically is assumed to result from the cell-culture systems used for assaying transfection, i.e., expression levels are artificially elevated because aggregated complexes more readily sediment onto adherent cell lines. However, our previous work with suspended cells has demonstrated that aggregation cannot explain the observed increases in transfection (84). In light of these findings, it is interesting that the lyophilization process has been shown to cause some denaturation of double-stranded DNA (53). This observation is particularly intriguing considering that recent work has suggested that partial denaturation of the promoter region can actually stimulate transcription (144). Although partial denaturation of naked DNA during dehydration would likely be reversed upon rehydration, strand separation could potentially be preserved in nonviral vectors if cationic agents rearrange to accommodate the altered structure. This notion is consistent with studies showing that lipids bind differently to DNA possessing different structures (145). Alternatively, dehydration might cause structural changes in the complex (e.g., increased encapsulation) that facilitate transfection (146). Although the increases in transfection that have been observed after freezing and drying are quite modest, such an effect complicates the assessment of recovery, but potentially could be used to bolster transgene expression *in vivo*.

The lyophilization process involves two stresses that are known to damage macromolecules, i.e., freezing and drying. Several studies have shown that slow cooling causes much more damage to vectors than rapid freezing (103,105,109). As discussed earlier, this effect is owing to the greater amount of time that vectors spend in the concentrated unfrozen solution prior to solidification when cooling rates are slow (68,84,105). Although the time available for coalescence in the unfrozen fraction can be altered significantly by the degree of supercooling, quick-freezing (e.g., plunging samples into liquid nitrogen) causes rapid heat removal and solidification that limits

Table 3
Lyophilization of Gene Delivery Systems

Delivery vehicle	References	Formulation	Freezing protocol	Storage
DOTAP/DOPE	(143)	0.5 M trehalose, sucrose, glucose	Liquid nitrogen	—
DMRIE/Cholesterol	(142)			
Transferrin/PEI	(142)	10% sucrose in HBS	Precooled shelf to -40°C	—
Transferrin/polylysine/adenovirus				
adenovirus				
PDMAEMA	(127,148)	10% sucrose, trehalose, maltose	Precooled shelf to -40°C	—
PDMAEMA	(102)	10% sucrose + 20 mM HEPES	Precooled shelf to -40°C ; stored at 20°C	10 mo; size, supercoil content, transfection retained
DOTAP/cholesterol/protamine retained	(101)	10% lactose, maltose, sucrose, trehalose	-20°C (30 min) then -80°C (30 min); -80°C (30 min); liquid nitrogen; stored at 25°C	8 wk; size + transfection
DOTAP/protamine/DNA retained	(141)	3% lactose	Liquid nitrogen or spray-dried, stored under vacuum, 25°C	12 wk; transfection
DMRIE/cholesterol	(107)	1%, 5% glucose, lactose, trehalose sucrose, mannitol, PEG, hydroxyethyl starch	-38°C at $-1^{\circ}\text{C}/\text{min}$	—
Alkylated cys-Typ-Lys ₁₈	(151)	5% sucrose, PEGylation	Dry ice	—
Polylysine/DNA	(142)	10% sucrose + HBS	Precooled shelf to -40°C , stored at -20°C	3 wk; transfection retained
Adeno-associated virus	(165)	0.4% sucrose 0.4% mannitol 1 mg/mL protamine	-40°C at $-1^{\circ}\text{C}/\text{min}$, stored at 25°C	3 mo; titer maintained

the exposure of vectors to the highly concentrated unfrozen fraction. Although rapid cooling rates are achievable in commercial lyophilization, problems associated with liquid nitrogen sterilization and handling makes this approach less desirable (147). Many studies have hastened freezing by loading filled vials on precooled shelves (102,127,142,148), but cooling rates under these conditions are still slow when compared to that achieved with liquid nitrogen or by immersion in an ethanol/dry ice bath (103,105,109,141). Accordingly, we must apply the knowledge gleaned from the cryoprotection experiments discussed previously to preserve vectors during the freezing step of the lyophilization process. However, it is critical that freezing studies mimic the cooling rates and incubation times encountered in a freeze-drying cycle if they are to be applicable to stability during lyophilization.

Although nature has perfected methods of protecting extremely complicated systems (i.e., whole organisms) during freezing and drying (149), the prospect of stabilizing multicomponent assemblies (e.g., nonviral 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 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 predominantly focused on relatively simple systems, e.g., purified proteins, 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 nonviral vectors (150). However, it is encouraging that lyophilization studies to date have reported acute stabilization of polymer-based, lipid-based, and viral vectors (Table 3).

Similar to the stabilization achieved in freeze-thaw studies, protection of gene-delivery systems during lyophilization has been reported when sugars are included in the formulation (101,102,107,127,141–143,148). It is clear that the choice of excipient is critical for protection during both the freezing and drying steps (107,108). However, the dilution of vectors in concentrated excipient solutions is problematic because gene delivery is most efficacious at high vector concentration (93,103). Although rehydration with reduced quantities of water can effectively re-concentrate 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 (84).

The mechanism by which excipients stabilize nonviral vectors during lyophilization has yet to be elucidated. In addition to stresses encountered during freezing (discussed previously), dehydration is known to perturb vector function (107). In the case of aqueous formulations of nonviral vectors, the removal of water eliminates the suspending medium and facilitates contact between particles in the dried state. The incorporation of excipients prior to lyophilization would allow particles to be separated in a matrix that could potentially be maintained in the dried state (108). This situation would minimize interactions between particles and inhibit aggregation. Apparently this separation can be maintained even if samples experience collapse, because we have repeatedly observed that collapse of the dried cake does not alter size or transfection rates upon rehydration (unpublished observations). This is consistent with a recent report by Kwok et al. showing that nonviral vectors can be stabilized without sugars during drying by incorporating high levels of PEGylated components into the particle (151). However,

similar experiments with PEGylated lipid-DNA complexes have shown clearly that formulation with stabilizing excipients is still necessary at lower PEGylation levels (99).

Although the recovery of particle size often correlates with transfection rates, interactions between the cationic agent and DNA within a complex can be disrupted during lyophilization without altering particle dimensions (125). Unfortunately, there is not more specific information about what structural characteristics are necessary for successful gene delivery, but it is clear that entrapment in a glassy matrix alone is unable to preserve macromolecular structure (125). Studies on dried liposomes and proteins have demonstrated that sugars are able to preserve structural integrity by forming hydrogen bonds with the macromolecule, thereby mimicking the “hydrated” condition (152–154). This concept has been termed the “water replacement hypothesis” by Crowe and colleagues (153,154), and has been implicated in the preservation of nonviral vectors in the dried state (101,107,143). Recent studies have clearly documented that lyophilization in the presence of sugars does not completely preserve transfection activity during dehydration despite the maintenance of particle size (99). The difference in the ability of sugars to protect complexes as compared with liposomes suggests that lipid-DNA complexes possess fundamentally different structures than traditional bilayers (156). Although obvious differences include the DNA and the cationic headgroup (as compared with anionic and neutral headgroups in traditional liposomes), lipids within complexes are known to adopt a nonbilayer, inverted hexagonal phase that causes exposure of hydrophobic moieties to the aqueous solution (106,131,155,156). Although the formation of such structures greatly facilitates membrane fusion and gene delivery, it is unclear whether water replacement via hydrogen bonding would be capable of stabilizing these hydrophobic assemblies. However, if vectors are designed such that the hexagonal phase is not induced until the vector fuses with a cellular membrane (as proposed in several studies (157–159), particles would be inherently more stable, and the water-replacement mechanism should operate according to that proposed for lipid bilayers (153,154). Clearly, more work is needed to understand the interactions between excipients and nonviral vectors so that lyophilized formulations can be rationally designed rather than empirically derived.

Considering the freezing and drying stresses discussed earlier, it is not surprising that preservation during lyophilization requires the use of stabilizing sugars (Table 3). Once in the dried state, it is typically assumed that vectors will be stable for indefinite periods of time. This naïve notion has caused leaders in the gene-therapy field to respond to questions about the shelf-life of their pet vector by stating: “It is lyophilized.” Although stability for 10 mo at room temperature in the dried state has been reported, two published studies have also investigated accelerated stability at slightly higher storage temperatures (101,102). In both of these studies, vectors stored at 37°–40°C exhibited decreased transfection rates as well as reduced supercoil content and increased particle size over the time course of the experiments. These results illustrate that degradation continues in the dried state, and that damage progressively accumulates even during storage at lower temperatures, albeit at slower rates. Our results from storage-stability experiments are consistent with this suggestion, and we observe a progressive degradation of lipid-DNA complexes in terms of transfection rates, supercoil content, particle size, and dye exclusion over 2 yr, even when samples are stored at –20°C (Molina et al., manuscript in preparation). Under conditions of prolonged storage, it is paramount to utilize nonreducing sugars (e.g., trehalose, sucrose), because reducing sugars (e.g., glucose, lactose, maltose) are known to form covalent adducts with nucleic acids (160). In

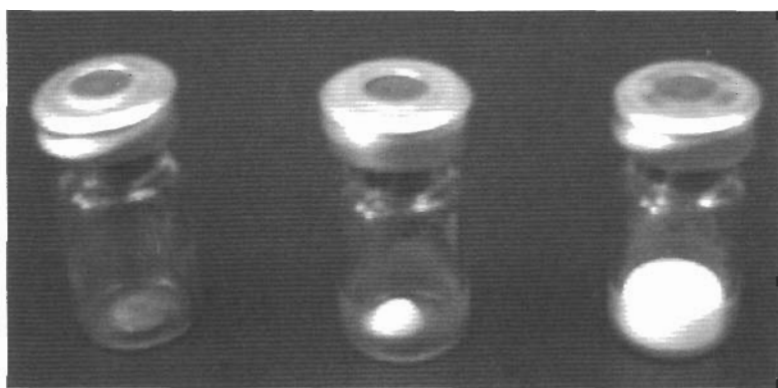


Fig. 5. Lyophilized formulations of lipid-DNA complexes stored at 60°C for 10 mo. Note the obvious discoloration in the glucose formulation (left) and collapse in the sucrose formulation (center). In contrast, only minor shrinkage is evident in the lyophilized trehalose cake (right).

our experience, prolonged storage in a reducing sugar produces DNA products that do not migrate into a 0.8% agarose gel, and results in a brown cake indicative of nonenzymatic glycosylation via the Maillard reaction (Fig. 5). Although this deterioration is visible in lyophilized samples stored at 40°C and 60°C, the same chemistry occurs at lower temperatures even though cakes do not become visually discolored (at least not after 10 mo). However, even when nonreducing sugars are employed, progressive changes in transfection, supercoil content, particle size, and dye exclusion can be observed. Considering that vectors are immobilized in the glassy excipient phase, these findings suggest that degradation can occur at significant rates despite maintenance of the glassy phase. Although molecular relaxation rates are greatly reduced in the glassy state, the irregular arrangement of molecules in the amorphous phase causes an increase in free volume that can facilitate diffusion (161). It follows that ROS generated during storage potentially could diffuse into and react with vectors in an amorphous matrix. Given that nucleic acids are known to be acutely sensitive to oxygen radicals (33), formulations should be designed to minimize the potential for generating ROS during prolonged storage. As suggested by Middaugh et al. for the formulation of naked DNA in aqueous solutions, the use of chelators and antioxidants may prove to greatly enhance the shelf-life of nonviral vectors (26). It should also be recognized that other vector components (e.g., lipids) are sensitive to oxidation, and that radicals generated from these components can subsequently react with the DNA (162–164). Given the ability of oxidized bases to mispair (33), deleterious reactions during storage can result in the *in vivo* production of mutated proteins with the potential for disastrous consequences; a situation that is unique to DNA-based therapeutics.

4. Conclusion

Aqueous formulations of nonviral vectors at the high concentrations necessary for clinical trials are very unstable, and it is doubtful that sufficient stability can be attained in liquid suspensions. Studies have shown that frozen formulations can offer prolonged stability in some cases, but freezing conditions and excipient mixtures that preserve one vector type do not necessarily stabilize other vectors (109,125). In addition, there are concerns associated with the potential for thawing and the costs of main-

taining the frozen state during shipping and storage. To date, lyophilization studies have reported that some nonviral vectors can be stabilized during acute freeze-drying stress, but prolonged storage studies are lacking. Unfortunately, most studies in this area have employed an empirical approach, and provide little insight into the mechanism(s) by which excipients stabilize DNA-based therapeutics. Future studies investigating the stability of nonviral vectors would be greatly enhanced if the interactions between cationic agents and DNA were better characterized such that stabilization strategies could focus on preserving specific nanoscale structures that are necessary for transfection. Considering the recent efforts toward understanding the subtle interactions within complexes (130–135), we hope that stability studies will soon be able to focus on the preservation of specific biophysical characteristics.

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