



RESEARCH ARTICLE

Development of formulations that enhance physical stability of viral vectors for gene therapy

MA Croyle¹, X Cheng¹ and JM Wilson²

¹The University of Texas at Austin College of Pharmacy, Division of Pharmaceutics, Austin, TX; and ²Institute for Human Gene Therapy and Department of Molecular and Cellular Engineering, University of Pennsylvania, Philadelphia, PA, USA

This study summarizes our initial efforts to address an issue that is critical to the success of any multicenter gene therapy clinical trial – maintenance of vector viability during shipping and storage at remote test sites. We have identified formulation and processing factors that influence stability of viral preparations such as selection of appropriate buffer systems, cryoprotectants, and storage conditions. Adenovirus and adeno-associated virus expressing E. coli beta-galactosidase (lacZ) were suspended in blends of complex carbohydrates, cyclodextrins and various surfactants. X-gal stains of 293 and 84-31 cells were used to determine infectious titer of all preparations. Potassium phosphate-buffered preparations consistently maintained high viral titers after storage at –20 and 4°C. Blends of sucrose, mannitol, and surfactant

showed negligible loss of titer for 35 days at 4°C. Formulations of sucrose and cyclodextrin were stable for 2 years at –20°C. Negligible loss in titer was observed in unit-dose viral preparations lyophilized in sucrose and stored at 4°C for 1 year after an initial loss of 0.5 log due to processing. Studies with lyophilized sucrose/mannitol blends have shown that viral recovery after processing is directly related to the final moisture content of the dried product. Virus concentration also plays a significant role in recovery after processing with highly concentrated preparations showing minimal loss in titer after lyophilization. In summary, lyophilized preparations that can be shipped and stored at 25°C offer a solution to the current problem of distribution of viral vectors for clinical trials. Gene Therapy (2001) 8, 1281–1290.

Keywords: adenovirus; AAV; physical stability; formulation; lyophilization

Introduction

Desired attributes of vectors for gene therapy include high affinity for the target tissue, suitable promoters to maximize gene expression, proper regulatory elements to synchronize transcription in response to biochemical stimuli and the ability to accommodate the desired transgene of interest.^{1–4} However, after exerting a significant effort to design an appropriate gene delivery vehicle, scientists generally place their novel vector in buffer, add glycerol and store the preparation at –80°C.⁵ While this has become standard practice, these conditions could have a detrimental effect on the overall function of a gene delivery system and are not conducive to use of the vector as a medicinal product. Vectors stored under these conditions must be quickly shipped to remote sites on dry ice, which is somewhat costly. This formulation also requires extensive dilution before administration to reduce the toxicity of glycerol.

The goal of the ultimate gene delivery system is to produce transgene expression with maximum safety, effectiveness and reliability. As described above, the molecular and biological aspects of the vector can simultaneously contribute to each of these objectives. The physical and chemical stability of the vector, however,

can also significantly affect each goal. As the vector degrades, transduction efficiency drops and the reliability and effectiveness of the preparation diminishes. If the degradation products are toxic or immunogenic, the safety of the preparation is also compromised. Optimization of formulations to enhance chemical and physical stability and prevent degradation of the gene delivery system over time will secure the reliability of the vector preparation. Formulations can also be developed to enhance the transduction efficiency and ultimately the effectiveness of the vector. Obviously, excipients employed in vector formulations must be suitable for use *in vivo*. Lastly, formulations can also promote vector safety as has recently been described with PEGylated viral preparations.^{6,7}

To date, most formulation efforts reported in the literature are focused on the addition of excipients to vector preparations to enhance transduction efficiency *in vivo*.^{8–11} Few reports describe the physical stability of viral vectors for gene therapy.^{12,13} This report is the first to summarize efforts to systematically develop formulations that enhance the physical and chemical stability of viral vectors at ambient temperature. We chose the adenovirus as our model vector, because it can easily be produced in quantities sufficient for real time assessment of viral stability. Studies were also initiated with adeno-associated virus, as this vector system is becoming increasingly popular for clinical applications. Excipients were selected from a panel of pharmaceutically acceptable compounds known to promote the physical stability of protein and

Correspondence: MA Croyle, The University of Texas at Austin, College of Pharmacy, Division of Pharmaceutics, PHR 4.214D, Austin, Texas 78712, USA

Received 20 February 2001; accepted 11 June 2001

peptide-based therapeutics currently on the market. For a comprehensive review of the principles and theory of the formulation of proteins, the authors refer the reader to a review by Wang.¹⁴ Critical factors that must be considered when developing formulations to enhance vector stability are identified. Important issues for the development of lyophilized preparations are also described. We believe this will serve as a useful reference for the molecular biologist interested in maximizing the reliability and physical stability of novel gene delivery systems before use in the clinic.

Results and discussion

Assessment of formulations for use in frozen and lyophilized viral preparations

Previous work demonstrated that adenovirus titer after a single freeze-thaw cycle was dependent upon the final frozen pH of the cryoprotectant medium.¹³ In this report, we assessed two buffers that are acceptable for human use – 10 mM sodium phosphate (DPBS) and 10 mM potassium phosphate (KPBS) buffered saline – for their ability to maintain pH at low temperature. The pH of sodium phosphate buffered saline dropped from 7.4 to 5.0 when frozen at -20°C and to pH 4 at -80°C (Figure 1a). Addition of glycerol to the buffer did not prevent the drop in pH upon freezing. Differential scanning calorimetry demonstrated that this change in pH is associated with precipitation of buffer components during freezing (Figure 1b).¹⁵ Addition of sucrose or trehalose to the buffer could not prevent this phenomenon at -80°C , but each sugar at a 1 M concentration did prevent dramatic pH changes at -20°C . Potassium phosphate buffer did not demonstrate the significant changes in pH as seen with the sodium phosphate buffer at -20°C , but did experience similar drops in pH at -80°C . Addition of cryoprotectants did not significantly affect the final pH of the preparation. However, it is important to note that for each buffer system, tertiary amine beta cyclodextrin (TMBCD) could maintain physiological pH upon freezing.

Virus was formulated in the manner described above and frozen at the respective temperature for 24 h. In

order to minimize loss due to rapid temperature changes,¹³ samples were thawed at 25°C and viral titer assessed in order to determine if change in pH would significantly affect physical stability. Adenoviral preparations were quite sensitive to pH changes upon freezing at the concentration studied (Figure 2a). Preparations that maintained initial pH (7.4) did not experience significant drops in titer upon thawing. Preparations that underwent a drop of 3 pH units suffered a one log loss of titer. Adeno-associated virus (AAV) was not as sensitive to pH changes upon freezing as the adenovirus at the concentrations studied (Figure 2b). AAV preparations that experienced a drop of 3 pH units upon freezing lost 0.5 log of infectious virus. As stated above, potassium phosphate buffered saline maintained pH to a higher degree than sodium phosphate buffered saline when frozen at -20°C . Adenovirus stored in 10% glycerol in this buffer system demonstrated superior stability over sodium phosphate buffered preparations (Figure 2c). As a result, potassium phosphate buffered saline was the buffer of choice for all formulations unless otherwise indicated.

Stability of Adenoviral Formulations at -20 and 4°C

After screening potential formulations and buffer systems for the ability to maintain pH at low temperature, large scale preparations of formulated virus were stored at -20 and 4°C for a period of 2 years. In order to assess the real-time stability of vector in each formulation, four to six vials from each lot were removed and assessed for transduction efficiency. Expiration dates were assigned to each preparation at the time when titer fell to 90% of its original value (ie suffered 10% loss).^{16–18} Glycerol preparations were the least stable at -20°C . The sodium phosphate buffered preparation lost 10% of the original titer in 2 days (Table 1). Potassium phosphate buffer extended the shelf life of the virus to 12 days. When glycerol was removed and sucrose added to the preparation, titer did not drop for approximately 1 month. Addition of cyclodextrins to the formulation extended shelf life to 360 (beta cyclodextrin) and 690 (tertiary amine beta cyclodextrin) days which is significantly longer than that of virus in glycerol at -80°C (approximately 37 days, unreported

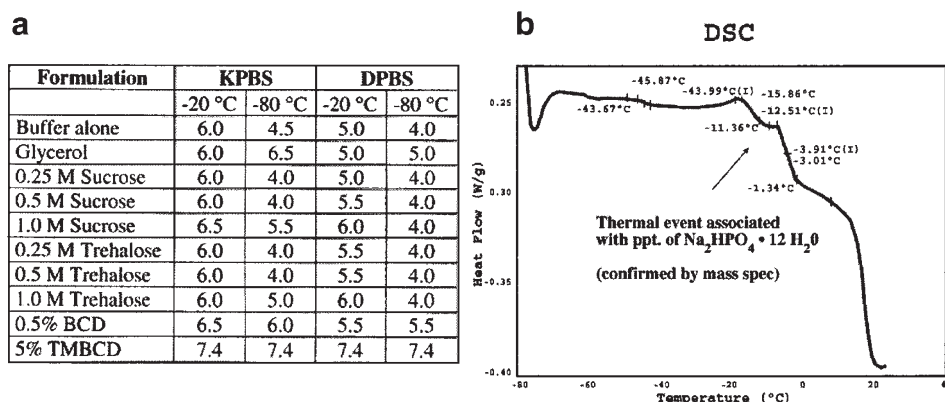


Figure 1 Addition of cryoprotectants to viral formulations prevents precipitation of buffer components and maintains physiological pH upon freezing. (a) Final frozen pH of proposed viral formulations at -20 and -80°C . All preparations were pH 7.4 before freezing. (b) DSC readout of the standard adenovirus formulation: sodium phosphate buffered saline and 10% glycerol. Mass spectroscopy revealed that the major thermal event detected in the middle of the scan (arrow) is associated with precipitation of the disodium hydrogen phosphate decahydrate component of the buffer which could be responsible for the dramatic change in pH detected upon freezing in this buffer. This event is absent in potassium phosphate buffered preparations.

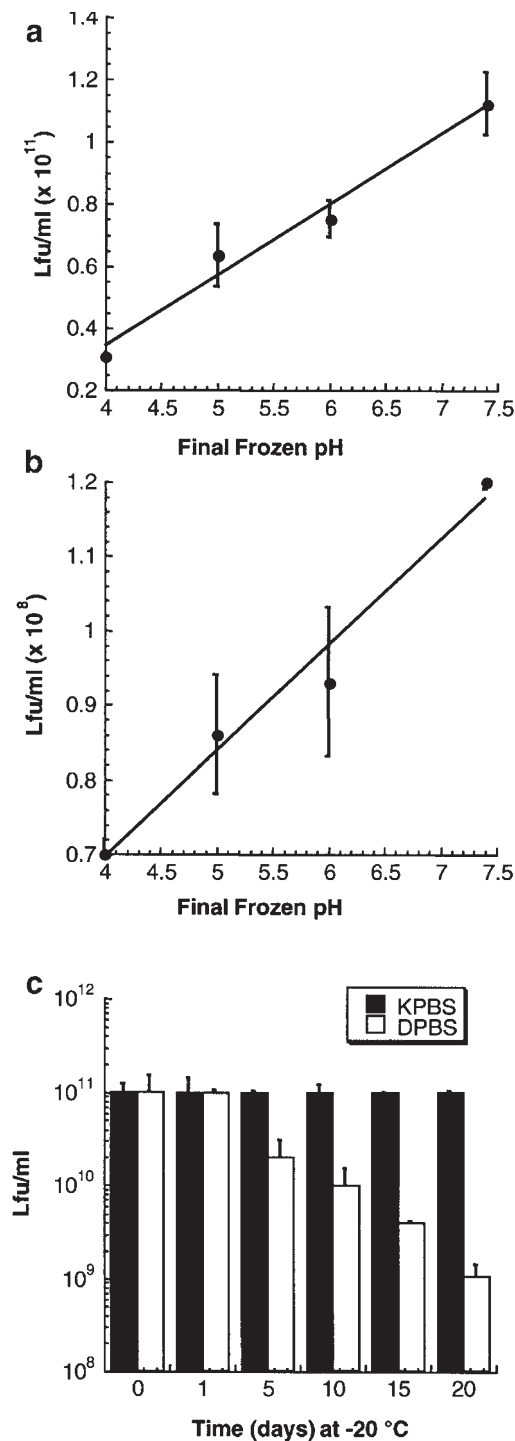


Figure 2 The final frozen pH of a formulation significantly affects the titer of a viral preparation upon thawing. Viral preparations were frozen at -20 and -80°C for 12 h. Viral titer was assessed and data grouped according to the final frozen pH of each formulation. (a) Adenovirus stability with respect to final frozen pH of the preparation (correlation coefficient, $r^2 = 0.98$). (b) AAV stability with respect to final frozen pH (correlation coefficient, $r^2 = 0.97$). (c) Stability profile of adenovirus prepared in either sodium (DPBS) or potassium (KPBS) phosphate buffered saline and 10% glycerol at -20°C . Results are reported as the average titer of 4–6 samples from two separate experiments. Error bars reflect the standard deviation of the data.

Table 1 Expiration dates for adenoviral formulations stored at -20°C

Formulation	Expiration date ^a
1 M Sucrose/KPBS/5% TMBCD	690 days
1 M Sucrose/KPBS/0.5% BCD	360 days
1 M Sucrose/KPBS	70 days
1 M Sucrose/DPBS/5% TMBCD	45 days
0.5 M Sucrose/KPBS	18 days
10% Glycerol/KPBS	12 days
10% Glycerol/DPBS	2 days
DPBS	12 h

^aExpiration dates represent the time for a preparation to lose 10% (0.1 log) of the original titer.

observation). All formulations stored at 4°C significantly enhanced viral stability (Table 2). Sucrose formulations containing beta cyclodextrin and surfactant did not display significant drops in titer for approximately 1 month.

Stability of AAV formulations

Initial stability studies with adeno-associated virus demonstrated that this virus is significantly more stable than the adenovirus. It took approximately 4 months for a preparation stored at 4°C in sodium phosphate buffered saline to lose 10% of the original titer (Table 3). Similar results were seen at 25°C . A formulation consisting of sucrose, mannitol, Span 20 and protamine extended AAV stability to 5 months at 4 and 25°C . Similar expiration dates were also noted with this formulation at -20 and -80°C .

Effect of formulation on transduction efficiency *in vivo*

Addition of any substance to a drug product can significantly alter the bioavailability of the active compound. Some excipients can enhance drug absorption.¹⁹ As a result, less medicine is needed to exert the desired effect. Some excipients, however, have been implicated as causing, contributing to, or playing some role in certain human reactions not related to the active compound. The Handbook of Food, Drug, and Cosmetic Excipients has reviewed many of these.²⁰ Thus, it was imperative that we evaluate formulations that significantly improved vector stability for their effect on transduction efficiency *in vivo*.

Because adenoviral vectors are suitable for many gene therapy applications, we studied the effect of these formulations on transduction efficiency after intramuscular, intravenous and intratracheal injection in C57BL/6 mice.

Table 2 Expiration dates for adenoviral formulations stored at 4°C

Formulation	Expiration date ^a
0.4% Sucrose/0.4% Mannitol/0.001% Span 20	35 days
1 M Sucrose/KPBS/BCD	20 days
1 M Sucrose/KPBS	10 days
1 M Sucrose/DPBS	8 days
0.5 M Sucrose/KPBS	7 days
0.25 M Sucrose/KPBS	2 days
DPBS	15 min

^aExpiration dates represent the time for a preparation to lose 10% (0.1 log) of the original titer.

Table 3 Expiration dates for adeno-associated virus (AAV) formulations

Formulation	−80°C	−20°C	4°C	25°C
0.4% Sucrose 0.4% Mannitol 0.01% Pluronic Protamine (0.5 mg/ml)	26.7 days	89.5 days	240 days	24.7 days
0.4% Sucrose 0.4% Mannitol 0.001% Span 20 Protamine (1.0 mg/ml)	149.4 days	149.65 days	150.1 days	153.5 days
DPBS 10% glycerol	32.4 days	37.4 days	ND	ND
DPBS	27 days	ND	135.4 days	125.5 days

Expiration dates represent the time for the preparation to lose 10% (0.1 log) of the original titer.
ND, stability not assessed at this temperature.

Transduction efficiency of formulated virus was compared with vector in phosphate buffered saline and the standard 2% glycerol preparation commonly used in pre-clinical testing of viral vectors.²¹ A 5% glycerol formulation was also included as a semi-toxic control by which to compare our formulations. A significant increase in transgene expression was detected after intramuscular injection of the sucrose/beta cyclodextrin and 5% glycerol formulations when compared with that seen in virus formulated in potassium phosphate buffered saline (Student's *t* test, $P \leq 0.01$, Figure 3a). The other formulations did not significantly effect gene expression in muscle and were well tolerated. While the sucrose/tertiary amine beta cyclodextrin formulation enhanced transduction efficiency of the virus in the lung, transduction efficiency was reduced by the 5% glycerol preparation (Figure 3b). This effect was not detected in animals given the other formulations. All formulations tested slightly enhanced transduction efficiency when administered intravenously.

Development of lyophilized viral vectors

Even though our formulation efforts significantly enhanced adenoviral stability at −20 and 4°C, they would still require shipment on ice. Lyophilization, a technique commonly employed to extend the chemical and physical stability of labile compounds at ambient temperatures, was considered as a practical alternative. The lyophilization process consists of three stages: freezing, primary drying and secondary drying (Figure 4). During the freezing step, samples are frozen in the range of −40 to −50°C. When the product is completely frozen, the pressure in the lyophilization chamber is reduced and heat is applied to the product. Under these conditions, water is removed by sublimation, a phase change from the solid state directly to the vapor state without the appearance of an intermediate liquid phase. This is the primary drying phase. After primary drying, additional drying is necessary to remove any water that remains in the product. During secondary drying, heat is slowly added to the highest allowable temperature to maintain product

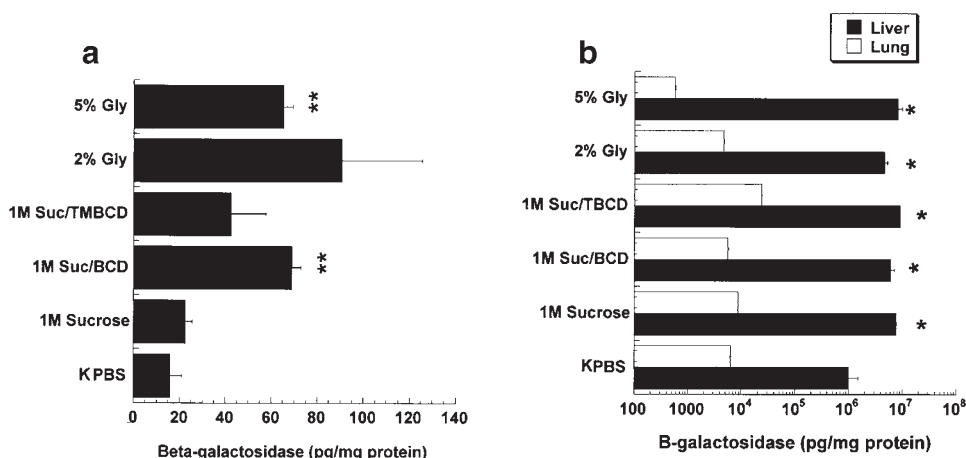


Figure 3 Formulations that enhance adenoviral stability do not affect transduction efficiency. C57BL/6 mice were given adenovirus in formulations that maintained viral stability at −20°C (See Table 1). 2% Glycerol is the standard formulation commonly used in pre-clinical testing of viral vectors. The 5% glycerol formulation was included as a standard by which to measure toxicity of the new formulations. (a) Transduction efficiency of formulated adenovirus in muscle. (b) Beta-galactosidase expression in liver (solid bars) and lung (open bars) after administration of formulated adenovirus. Data are average beta-galactosidase levels from groups of five animals. Error bars represent the standard deviation of the data. * $P \leq 0.05$, ** $P \leq 0.01$, Student's *t* test.

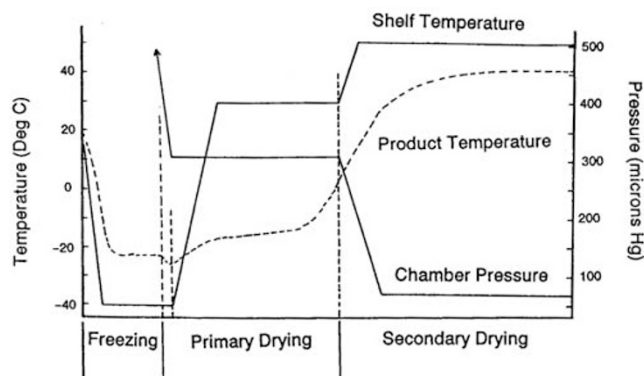


Figure 4 Process variables during a representative lyophilization cycle.

viability and maintained at this level until the process is complete.

Many factors affect product recovery during the lyophilization process. Selection of appropriate excipients that promote stability upon freezing, drying and storage are critical for the success of any lyophilization process.^{22–26} A large batch of adenovirus was purified and divided into five separate groups. Each group was desalted into different formulations. Excipients were selected according to their ability to replace water and form a protective shell around the viral capsid in order to maintain protein conformation during the freezing and drying process as described previously.^{27–30} The initial titer of the lot was 1×10^{11} lfu/ml. One milliliter aliquots were placed in glass vials and lyophilized under a standard protocol (-40°C for 2 h, -33°C for 12 h, -25°C for 5 h, 10°C for 4 h and 20°C for 8 h at a pressure of 30 mtorr). Preparations lyophilized in sodium or potassium phosphate buffer alone suffered significant losses of 5 and 4 logs of infectious virus respectively (Figure 5). When sucrose, mannitol and Span 20 were added to formulations, titer fell by approximately 1 log. Titer fell by less than 1 log in preparations lyophilized in 1 M sucrose.

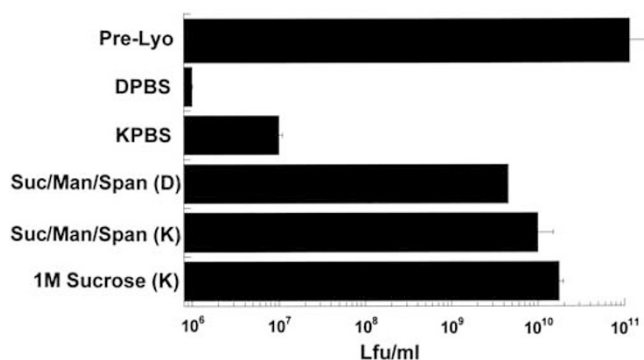


Figure 5 Addition of excipients to adenoviral preparations significantly affects recovery of active vector after lyophilization. Large-scale adenovirus preparations were divided into five groups, desalted into various formulations, placed in glass vials and lyophilized under a standard protocol (see text). Pre-lyophilization (Pre-lyo) titer for the entire lot was 1×10^{11} lfu/ml. Concentrations of sucrose, mannitol and Span 20 in the Suc/Man/Span formulations are 40 mg/ml, 40 mg/ml and 0.001% respectively. K, KPBS; D, DPBS. Data are the average of eight vials from two separate experiments. Error bars represent the standard error of the data.

Effect of viral concentration on glass transition temperature (T_g') of formulations for lyophilization

Even though all excipients significantly improved recovery of adenovirus after lyophilization, each preparation still suffered a drop in titer, which we attribute to the process itself. Thus, formulations were further characterized in order to maximize viral recovery upon completion of the lyophilization cycle. One parameter that is important for ascertaining the required processing conditions is the glass transition temperature (T_g'), the temperature of maximum freeze concentration where, in a frozen solution, the residual non-ice phase forms a glass.³¹ If primary drying is carried out above T_g' , the freeze concentrate behaves as a viscous liquid, the microstructure of ice formed upon freezing will be lost and product recovery is significantly hampered. If drying occurs below the T_g' , water will be rapidly removed without disrupting the microstructure established upon freezing which minimizes product loss during lyophilization.³² Thus, the T_g' of a preparation sets the safe upper temperature limit for primary drying.

Because T_g' can vary over an enormous range (-1 to -50°C), determination of this value for a proposed formulation is extremely important and the first step in process development. The T_g' of a preparation is very concentration-dependent: buffer salts, amino acids, carbohydrates and other components added to a formulation can influence T_g' .³³ Differential scanning calorimetry (DSC) gives a direct measurement of T_g' .^{34–36} A representative thermogram is depicted in Figure 6a. Sucrose (1 M) in potassium phosphate buffered saline has a glass transition of -34.5°C (Figure 6b). When adenovirus was added at a concentration of 1×10^{11} particles/ml, the T_g' rose to -33.9°C . Addition of another log of virus increased the glass transition temperature to -32.0°C . This effect is commonly seen with proteins.^{22,37} As protein replaces the water in a preparation, the glass transition rises. Adeno-associated virus had a different effect on the T_g' of a 0.5 M sucrose formulation. Addition of the virus at a concentration of 1.55×10^{11} genome copies/ml lowered the glass transition temperature from -33.5 to -34.71°C . Addition of another log of virus dropped T_g' to -35.37°C . These results suggest that this virus may interact with excipients in the frozen state differently than traditional proteins. Further evaluation of this phenomenon is currently underway in our laboratories. Based on this information, the primary drying temperature for the Ad preparation was set at -35°C . AAV was dried at -38°C .

Effect of final moisture content of the lyophilized cake on the recovery of adenovirus

One of the most critical factors when developing a lyophilized product is the level of residual water in the final product. For many protein preparations, the lower the residual moisture, the more stable the product.^{38–40} Pristoupil et al⁴¹ speculate that residual water disrupts excipient-protein interactions which stabilize conformation in the dry state and therefore interferes with the conformation protection offered by the formulation. It is also possible that excessive water in a preparation promotes conformational flexibility of the protein and the additional water has the ability to mobilize potential reactants in the semi-solid system facilitating chemical degradation and rendering the product unstable.^{42,43}

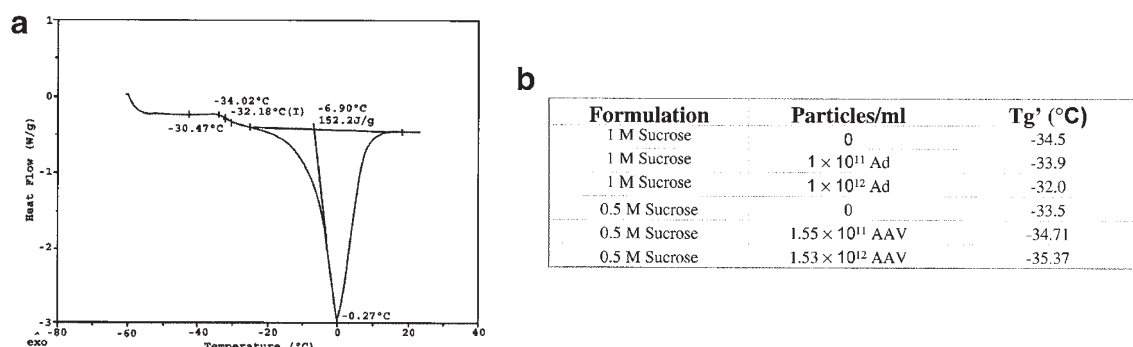


Figure 6 Viral concentration affects the glass transition temperature (T_g') of formulations for lyophilization. (a) Representative thermogram for an adenovirus preparation in 1 M sucrose. (b) Glass transitions of representative viral formulations. Addition of adenovirus to a preparation increased the glass transition temperature. AAV had an opposite effect.

The concept that overdrying a protein will cause loss of activity, although theoretically possible, has little experimental support in the literature. However, Grieff^{44,45} clearly demonstrated that, for influenza virus, stability is optimal at an intermediate water level (1.7%) and that over-drying could promote disruption and aggregation of viral particles, rendering them uninfected. Keeping this information in mind, we tested the ability of various blends of sucrose and mannitol for final moisture content after a standard lyophilization cycle (−40°C for 2 h, −35°C for 11 h, −10°C for 2 h, 0°C for 2 h and 25°C for 3 h at a pressure of 30 mtorr). When adenovirus was added to these preparations at a concentration of 5.12×10^{11} lfu/ml, recovery of infectious virus was extremely sensitive to the final moisture content of the product (Table 4). A preparation of 1:4 sucrose:mannitol had a final moisture content of 0.6% and suffered a 1.1 log loss in viral titer. The 3:4 ratio, with a moisture content of 1.3%, lost less than 1 log of infectious virus. A preparation consisting of a 1:1 ratio of sucrose:mannitol did not experience any loss of titer during the entire lyophilization process. The moisture content of this preparation was 1.4%. If water content rose 0.2% beyond this to 1.6%, titer fell by 2.2 log units. These data indicate that adenovirus is not only sensitive to the final water content in the lyophilized product, but that there is a rather narrow window in which viral titer can be maintained. As a result, all adenoviral lyophilization processes were tailored to produce a final moisture content of 1.3–1.5% in order to afford maximum recovery of virus.

Effect of virus concentration on recovery of viral titer after lyophilization

If a product still suffers loss in activity after extensive formulation and process development, it is common practice to compensate for the loss by adding additional compound to the preparation before lyophilization.^{16,46} While this practice usually produces products with the desired activity, additional protein could be detrimental to the overall stability of the product particularly during primary drying. High concentrations of protein may induce aggregate formation and precipitation upon freezing resulting in poor recovery of the product. In order to assess the maximum allowable concentration of adenovirus that facilitates optimal recovery of viral titer after lyophilization, adenovirus was lyophilized at two different concentrations in two separate formulations to a final moisture content of approximately 1.4%. This was done to assure that we could truly assess the effect of viral concentration on recovery in the absence of moisture effects (Table 5). Preparations at low viral concentration ($2\text{--}5 \times 10^{10}$ lfu/ml) suffered a loss of approximately one log of virus regardless of formulation. The loss in titer was less than one log at higher adenovirus concentrations ($2\text{--}5 \times 10^{11}$ lfu/ml). We believe that this is in part due to the ability of the virus itself to assist in maintaining the final frozen pH of a preparation upon freezing at high concentrations (data not shown). Adenovirus preparations of $1\text{--}5 \times 10^{11}$ lfu/ml were used in additional lyophilization studies.

Table 4 Final moisture content of the lyophilized cake significantly affects recovery of adenovirus

Sucrose:Mannitol Ratio	Pre-lyo titer (lfu/ml)	Post-lyo titer (lfu/ml)	Moisture content
1:4	$5.12 \pm 0.01 \times 10^{11}$	$4.6 \pm 0.2 \times 10^{10}$	$0.6 \pm 0.01\%$
1:2	$5.12 \pm 0.01 \times 10^{11}$	$2.8 \pm 0.11 \times 10^{11}$	$0.9 \pm 0.001\%$
3:4	$5.12 \pm 0.01 \times 10^{11}$	$4.77 \pm 0.04 \times 10^{11}$	$1.3 \pm 0.07\%$
1:1	$5.12 \pm 0.01 \times 10^{11}$	$5.08 \pm 0.1 \times 10^{11}$	$1.4 \pm 0.25\%$
2:1	$5.12 \pm 0.01 \times 10^{11}$	$2.89 \pm 0.3 \times 10^{11}$	$1.6 \pm 0.1\%$
4:1	$5.12 \pm 0.01 \times 10^{11}$	$1.0 \pm 0.22 \times 10^{11}$	$3.35 \pm 0.05\%$

The 1:1 formulation consisted of 10 mg/ml sucrose and 10 mg/ml mannitol. Changes in this ratio were made based upon this original formulation, ie the 3:4 ratio consists of 30 mg/ml sucrose and 40 mg/ml mannitol, the 1:2 ratio is 10 mg/ml sucrose, 20 mg/ml mannitol, etc. Data are the average of 10 vials from a single lyophilization run.

Table 5 Effect of initial adenoviral concentration on the recovery of infectious virus after lyophilization

Formulation sucrose:mannitol ratio	Pre-lyo titer (lfu/ml)	Post-lyo titer (lfu/ml)
1:4	$5.2 \pm 0.04 \times 10^{10}$	$4.6 \pm 0.01 \times 10^9$
1:4	$5.5 \pm 0.1 \times 10^{11}$	$2.97 \pm 0.1 \times 10^{11}$
4:1	$1.9 \pm 0.2 \times 10^{10}$	$1.0 \pm 0.02 \times 10^9$
4:1	$2.0 \pm 0.14 \times 10^{11}$	$1.6 \pm 0.1 \times 10^{11}$

Preparations with higher initial titers produced better recovery of active virus after lyophilization. Results are the averages of 10 vials of each preparation.

Stability of lyophilized viral vectors

After careful evaluation of process and formulation effects, a large-scale preparation of adenovirus (1×10^{11} lfu/ml) was lyophilized in a 1 M sucrose formulation under the following protocol: -40°C for 2 h, -35°C for 9 h, -10°C for 4 h, 0°C for 4 h and 25°C for 8 h at a pressure of 30 mtorr. After lyophilization, some vials were reconstituted. The only loss detected in this preparation after 1 year at 4°C was the initial loss of 0.5 log of virus due to the process itself (Figure 7a). Virus from the same lot prepared in 10% glycerol and stored at -20°C suffered a drop in titer of approximately 2 logs over the same time period. We also assessed the stability of reconstituted preparations stored at 4°C (Figure 7b). Virus titer dropped at a steady rate of 0.5 log/day over a period of 4 days, indicating that the vector must be used soon after reconstitution. This degradation rate was similar for other lyophilized preparations regardless of formulation (data not shown). Studies to elucidate the nature of this rapid decline in titer and develop novel admixtures for reconstitution which promote viral stability are currently underway.

Little work was done with respect to the development of formulations and optimization of the lyophilization process for AAV vectors. In a single experiment, half of an AAV preparation (8.7×10^8 lfu/ml) was desalted into potassium phosphate buffered saline. The remaining half was desalted in a formulation of 0.4% sucrose, 0.4% mannitol and protamine. Each preparation was lyophilized under the following protocol: -40°C for 2 h, -38°C for 11 h, -10°C for 2 h, 0°C for 2 h, and 25°C for 3 h at a pressure of 30 mtorr. After an initial loss of 0.3 log in titer due to the lyophilization process, the formulated preparation did not experience any loss in infectivity for 90 days at 25°C (Figure 8). It is also important to note that the preparation prepared in buffer without any additional excipients (buffer) experienced a similar loss in titer after lyophilization. This suggests that AAV may be resistant to the stresses of freeze-drying and pose less of a challenge to the formulation scientist with respect to stability.

Development of formulations that enhance vector stability under certain storage conditions is crucial for the success of any gene therapy program. This report provides some insight into factors that affect the chemical and physical stability of viral vectors for gene therapy. The final frozen pH of a formulation significantly affects the titer of both adenovirus and adeno-associated virus preparations. However, this effect can be influenced by

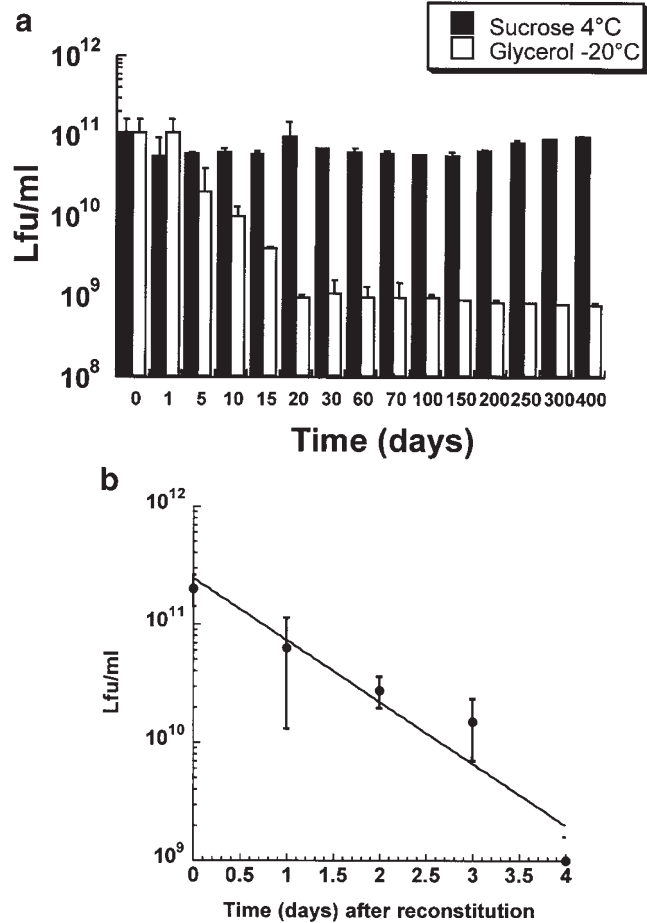


Figure 7 Stability of lyophilized adenoviral preparations. (a) Stability profile of adenovirus lyophilized in 1 M sucrose and stored at 4°C and a preparation from the same batch stored in 10% glycerol at -20°C . Data are the average titer from 10 vials of virus. Error bars reflect the standard deviation of the data. (b) Stability profile of reconstituted virus stored at 4°C . Data represent the average titer from approximately 20 vials of virus. Error bars reflect the standard deviation of the data. Similar rates of degradation were seen with other formulations upon reconstitution (data not shown).

other factors such as the purity of the preparation, initial starting concentration of virus and the rate at which the virus is frozen and thawed. Proper buffer selection and addition of certain excipients to a preparation such as sucrose and cyclodextrins prevent fluctuations in pH upon freezing, and can maintain viral titer for periods of over 1 year at -20°C . Lyophilization of vector preparations can afford remarkable stability at 25°C . Factors that influence viral recovery after lyophilization are excipient selection, vector concentration and residual moisture content of the final dried preparation. Data from AAV preparations suggest that each vector may have its own unique stability profile. In addition, modification of vectors within a group (ie adenovirus deleted of E1 and E4 vs a 'gutless' adenovirus deleted of all virus sequences) may possess different stability profiles from those described here. All most certainly will be different than non-viral vectors, each of which will also have distinct stability profiles.

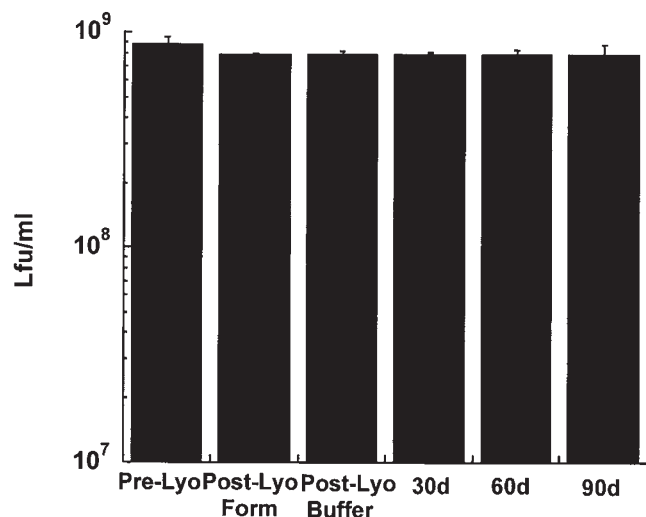


Figure 8 Stability of lyophilized AAV preparations. AAV was desalted into either potassium phosphate buffered saline (Buffer) or a formulation of 0.4% sucrose, 0.4% mannitol and protamine (1 mg/ml) and lyophilized under a standard protocol. Data are the average titer of 10 vials of each preparation. Error bars represent the standard deviation of the data.

Materials and methods

Materials

Sucrose USP, beta cyclodextrin, D-mannitol USP, D(+) trehalose, sorbitan monolaurate (Span 20), and phosphate-buffered saline (PBS) were purchased from Sigma (St Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and potassium chloride USP were purchased from JT Baker (Phillipsburg, NJ, USA). Glycerol USP was purchased from EM Science (Gibbstown, NJ, USA). Tertiary amine beta cyclodextrin was purchased from Cerestar USA (Hammond, IN, USA) (current supplier: CarboMer, Westborough, MA, USA). Pluronic block copolymer F68 was kindly provided by the BASF corporation (Mt Olive, NJ, USA). Universal pH indicator was purchased from Fisher Scientific (Pittsburgh, PA, USA).

Preparation of adenovirus

First-generation adenovirus expressing *E. coli* β -galactosidase under the control of a CMV promoter was amplified in 293 cells using a modification of established methods.⁴⁷ Virus was purified from cell lysates by banding twice on CsCl gradients followed by desalting on Econo-Pac 10 DG disposable chromatography columns (BioRad, Hercules, CA, USA) equilibrated with each respective formulation. Concentration of the virus was determined by UV spectrophotometric analysis at 260 nm and lac-forming assays (see below). All experiments were performed with freshly purified adenovirus.

Preparation of adeno-associated virus

Production of recombinant AAV2 expressing the *E. coli* beta-galactosidase gene under the control of a CMV promoter involved transfecting 60% confluent 293 cells in 150 mm dishes with a 1:1:2 ratio of *cis* plasmid (pAAVlacZ, with AAV ITRs), *trans* plasmid (p5E18, containing AAV2 *Rep* and AAV1 *Cap*)⁴⁸ and helper plasmid (p Δ 13, an adenovirus plasmid with most of the late

genes deleted and an 8 kb deletion in the E2b region). Transfection was performed by calcium phosphate precipitation. Ninety-six hours after transfection, cells were harvested and subjected to two rounds of CsCl gradient purification as described previously.⁴⁹ Virus was desalted by dialysis against respective formulations.

Sample preparation

All formulations were prepared under GLP conditions and sterilized by filtration through a 0.22 μm filter (Corning). For all studies, 500–1000 μl aliquots were added to autoclaved 3 ml clear borosilicate glass vials (Wheaton, Millville, NJ, USA). Vials were topped with 13 mm gray butyl rubber stoppers (Wheaton) and sealed with tear-off aluminum seals (Wheaton). For pH studies, 10 μl of universal pH indicator was added to monitor pH changes during freezing.

pH studies

Eight samples were prepared for each formulation as described. Four samples contained universal pH indicator.^{13,50} The remaining samples did not contain the indicator and were used to determine viral titer after freezing. Samples were frozen for 24 h at -20 and -80°C . pH of the frozen products were determined visually and noted for each formulation. Samples not containing indicator were slowly warmed to 25°C and assessed for viral potency.

Stability studies

Samples were prepared as described previously. Prior to storage at -20 or 4°C ($t = 0$), viral titer was determined by lac-forming assay. At each time point, four to six vials were assessed for viral infectivity. Viral titer of each formulation was compared with titer from virus produced in the same lot stored at -20°C in KPBS.

Lyophilization

For each lyophilization study, a lot of virus was divided into two groups with one half desalted in the desired formulation and the other desalted in DPBS containing 10% glycerol. The latter preparation was stored at -20°C and served as a control by which to assess the ability of lyophilization to enhance viral stability. Viral titers were assessed before storage at -20°C and lyophilization ($t = 0$). Vials containing the formulated preparation were placed in a single shelf research grade lyophilizer (FTS Systems, Stone Ridge, NY, USA) and cooled at a rate of $1^\circ\text{C}/\text{min}$ to -40°C . Three vials of formulation without virus were fitted with lead platinum RTD temperature probes (FTS) and placed in the front, middle and back of the lyophilization unit to monitor product temperature changes during the process. After cooling, all samples were lyophilized according to methods tailored to the specific formulation under study.³⁷ After drying was complete, samples were stoppered under vacuum and sealed with 13 mm tear-off aluminum seals (Wheaton). Samples were stored at 4°C until assayed for viral titer. Lyophilized virus was reconstituted with 1 ml sterile water for injection USP and assayed with samples stored at -20°C in DPBS and 10% glycerol.

Differential scanning calorimetry (DSC)

DSC analysis was performed with a MDSC 2920 (TA Instruments, New Castle, DE, USA) equipped with liquid

nitrogen cooling. The calorimeter was calibrated for temperature and cell constant using indium. Fifty microliters of each formulation were analyzed in hermetically sealed aluminum pans (TA Instruments). The cooling rate was 1°C/min. The temperature range was -60 to +25°C. Glass transition values (T_g') are reported as the midpoint of the observed transition. Experiments were performed in duplicate.

Moisture analysis

Water content of lyophilized formulations was determined by Karl Fischer titration⁵¹ and confirmed by thermogravimetric analysis (TGA).⁵² For Karl Fischer titration, an Aqua Star Karl Fischer titrator (Model C3000, EM Sciences) equipped with a fritless cell was calibrated with Karl Fischer water standard (2-methoxy ethanol) and blanked with the addition of 1 ml anhydrous methanol (EM Sciences). Lyophilized samples were reconstituted with anhydrous methanol and added to the titrator. TGA was performed with a Perkin-Elmer TGA 7 Series thermogravimetric system. Three to five milligrams of lyophilized preparation was scanned at 10°C/min from 25 to 200°C.

Limiting dilution/lac-forming assays

The term lac-forming unit (lfu) defines the number of infectious viral particles present in a preparation of beta-galactosidase (*lacZ*) expressing virus as measured by limiting dilution, infection of 293 cells (or 84-31 cells, a cell line expressing adenovirus E1 and E4 in the case of AAV),⁵³ histochemical staining and visual identification of beta-galactosidase positive (*lac*⁺) cells. Samples were serially diluted in DMEM supplemented with 2% fetal bovine serum. Medium was removed from 12-well plates seeded with 1.5×10^4 cells/well and 0.2 ml of the appropriate dilution was placed on the monolayers. After 2 h at 37°C, 2 ml of complete medium was added to each well and the infection continued for 16 h at 37°C. At this time, medium was removed and cells stained for beta-galactosidase expression as described previously.⁵⁴ *Lac*⁺ cells were tallied from a minimum of 20 microscope fields (approximately 48000 cells). Lac-forming units were calculated as described previously.¹³ The sensitivity of the assay is 10 to 1×10^{13} lfu/ml.

In vivo testing of formulated virus

C57BL/6 mice (6–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Preparations were administered either via the tail vein (1×10^{11} particles in 100 μ l formulation), intratracheally (5×10^{10} particles in 50 μ l), or intramuscularly (5×10^{10} particles in 50 μ l). Four animals from each group were necropsied either 4 days (intravenous and intratracheal) or 7 days (intramuscular) after injection. Tissues were homogenized in 1 ml of lysis buffer using a Brinkman polytron. Extracts were centrifuged at 14000 r.p.m. for 10 min. Protein concentration of supernatants was determined with BioRad DC protein assay reagents and bovine serum albumin as a standard. Extracts were quick-frozen in dry ice and stored at -80°C until assayed. β -Gal concentrations were determined with an ELISA kit (Boehringer-Mannheim) according to manufacturer's instructions. Blood samples were collected from remaining animals of each group via the retro-orbital sinus 4 and 7 days after injection for assessment of transaminase levels by an out-

side contract lab (Antech, New York, NY, USA). All animals were bled before initiation of the study for assessment of baseline transaminases.

Acknowledgements

The authors sincerely thank Dr Andrew McGee and Mr William Romanow of the University of Pennsylvania Department of Material Science Engineering and the Laboratory for Research on the Structure of Matter for assistance with differential scanning calorimetry and thermogravimetric analysis. We would also like to thank Animal Models Group (Marcia Houston-Leslie, Rosalind Barr, Jeanna Stabinski, Holly Clouse) of the Institute for Human Gene Therapy for expert technical assistance with vector administration. This work was funded by the Cystic Fibrosis Foundation, the NIH (NHLBI P01 HL59407, NIDDK P30 DK47757), and Genovo, Inc., a biotechnology company which Dr Wilson founded and has equity in. MAC is a recipient of a National Research Service Award.

References

- 1 Pap T, Gay RE, Gay S. Gene transfer: from concept to therapy. *Curr Opin Rheumatol* 2000; **12**: 205–210.
- 2 Nettelbeck DM, Jerome V, Muller R. Gene therapy: designer promoters for tumour targeting. *Trends Genet* 2000; **16**: 174–181.
- 3 Hart IR. Tissue specific promoters in targeting systemically delivered gene therapy. *Semin Oncol* 1996; **23**: 154–158.
- 4 Clackson T. Regulated gene expression systems. *Gene Therapy* 2000; **7**: 120–125.
- 5 Boyd JE. Facilities for large-scale production of vectors under GMP conditions. In: Meager A (ed.). *Gene Therapy, Technologies, Applications and Regulations*. John Wiley: Chichester, 1999, pp 383–400.
- 6 O'Riordan CR et al. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo*. *Hum Gene Ther* 1999; **10**: 1349–1358.
- 7 Croyle MA, Chirmule N, Zhang Y, Wilson JM. 'Stealth' adenoviruses blunt cell mediated and humoral immune responses against the vector and allow for significant gene expression upon re-administration. *J Virol* 2000; **75**: 4792–4801.
- 8 Marshall J et al. Cationic lipid structure and formulation considerations for optimal gene transfection of the lung. *J Drug Target* 2000; **7**: 453–469.
- 9 Wang G et al. Increasing epithelial junction permeability enhances gene transfer to airway epithelia *in vivo*. *Am J Respir Cell Mol Biol* 2000; **22**: 129–138.
- 10 Lanuti M et al. Use of protamine to augment adenovirus-mediated cancer gene therapy. *Gene Therapy* 1999; **6**: 1600–1610.
- 11 Croyle MA et al. Beta-cyclodextrins enhance adenoviral-mediated gene delivery to the intestine. *Pharm Res* 1998; **15**: 1348–1355.
- 12 Wold SM (ed.). *Adenovirus Methods and Protocols. Methods in Molecular Medicine*. Humana Press: Totowa, New Jersey, 1999.
- 13 Croyle MA et al. Factors that influence stability of recombinant adenoviral preparations for human gene therapy. *Pharm Dev Technol* 1998; **3**: 373–383.
- 14 Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm* 1999; **185**: 129–188.
- 15 Murase N, Franks F. Salt precipitation during the freeze-concentration of phosphate buffer solutions. *Biophys Chem* 1989; **34**: 293–300.
- 16 Carstensen JT. *Drug Stability: Principles and Practices, 2nd edn, Drugs and the Pharmaceutical Sciences*, vol. 68. Marcel Dekker: New York, 1995.
- 17 International Conference on Harmonization (ICH). *Harmonized Tripartite Guideline, Stability Testing of New Drug Substances and Products*. 1993.

- 18 Center for Drugs and Biologics FDA, Guidelines. *Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics*. Rockville, MD, 1987.
- 19 Hoener BA, Benet LZ. Factors influencing drug absorption and drug availability. In: Banker GS, Rhodes CT (eds). *Modern Pharmaceutics*. Marcel Dekker: New York, 1996, pp 121–153.
- 20 Smolinske SC. *Handbook of Food, Drug, and Cosmetic Excipients*. CRC Press: Boca Raton, FL, 1992.
- 21 Borellini F, Ostrove JM. The transfer of technology from the laboratory to the clinic: in process controls and final product testing. In: Meager A (ed.) *Gene Therapy Technologies, Applications and Regulations*. John Wiley: Chichester, 1999, pp 359–373.
- 22 Pikal MJ. Freeze-drying of proteins, part II: formulation selection. *Biopharm* 1990; **3**: 28–31.
- 23 Pikal MJ, Dellerman KM, Roy ML, Riggin RM. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm Res* 1991; **8**: 427–437.
- 24 Yoshioka S et al. Molecular mobility of protein in lyophilized formulations linked to the molecular mobility of polymer excipients, as determined by high resolution ¹³C solid-state NMR. *Pharm Res* 1999; **16**: 35–40.
- 25 Mattern M, Winter G, Kohnert U, Lee G. Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. *Pharm Dev Technol* 1999; **4**: 199–208.
- 26 Sun WQ, Davidson P, Chan HS. Protein stability in the amorphous carbohydrate matrix: relevance to anhydrobiosis. *Biochim Biophys Acta* 1998; **1425**: 245–254.
- 27 Carpenter JF, Crowe JH. The mechanism of cryoprotection of proteins by solutes. *Cryobiology* 1988; **25**: 244–255.
- 28 Crowe JH et al. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. *Cryobiology* 1990; **27**: 219–231.
- 29 Carpenter JF, Crowe JH. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* 1989; **28**: 3916–3922.
- 30 Timasheff SN. The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Ann Rev Biophys Biomol Struct* 1993; **22**: 67–97.
- 31 Franks F. Freeze-drying: from empiricism to predictability. *Cryo-Letters* 1990; **11**: 93–110.
- 32 Franks F. Freeze-drying: from empiricism to predictability: the significance of glass transitions. *Dev Biol Stand* 1991; **74**: 9–19.
- 33 te Booy MP, de Ruiter RA, de Meere AL. Evaluation of the physical stability of freeze-dried sucrose-containing formulations by differential scanning calorimetry. *Pharm Res* 1992; **9**: 109–114.
- 34 Her LM, Nail SL. Measurement of glass transition temperatures of freeze-concentrated solutes by differential scanning calorimetry. *Pharm Res* 1994; **11**: 54–59.
- 35 Hatley RH. The effective use of differential scanning calorimetry in the optimization of freeze-drying processes and formulations. *Dev Biol Stand* 1992; **74**: 105–122.
- 36 Hatley RH, Franks F. Applications of DSC in the development of improved freeze-drying processes for labile biologicals. *J Thermal Anal* 1991; **37**: 1905–1914.
- 37 Pikal MJ. Freeze-drying of proteins. Part 1: process design. *Biopharm* 1990; **9**: 18–27.
- 38 Pikal MJ, Dellerman K, Roy ML. Formulation and stability of freeze-dried proteins: effects of moisture and oxygen on the stability of freeze-dried formulations of human growth hormone. *Dev Biol Stand* 1992; **74**: 21–37.
- 39 Towns JK. Moisture content in proteins: its effects and measurement. *J Chromatogr* 1995; **705**: 115–127.
- 40 Jiang S, Nail SL. Effect of process conditions on recovery of protein activity after freezing and freeze-drying. *Eur J Pharm Biopharm* 1998; **45**: 249–257.
- 41 Pristoupil TI, Kramlova M, Fortova H, Ulrych S. Haemoglobin lyophilized with sucrose: the effect of residual moisture on storage. *Haematologia* 1985; **18**: 45–52.
- 42 Fitter J. The temperature dependence of internal molecular motions hydrated and dry alpha-amylase: the role of hydration in water in the dynamical transition of proteins. *Biophys J* 1999; **76**: 1034–1042.
- 43 Bell LN, Hageman MJ, Bauer JM. Impact of moisture on thermally induced denaturation and decomposition of lyophilized bovine somatotropin. *Biopolymers* 1995; **35**: 201–209.
- 44 Greiff D. Stabilities of suspensions of influenza virus dried by sublimation of ice *in vacuo* to different contents of residual moisture and sealed under different gases. *Appl Microbiol* 1970; **20**: 935–938.
- 45 Grieff D. Protein structure and freeze-drying: the effects of residual moisture and gases. *Cryobiology* 1971; **8**: 145–152.
- 46 Center for Biologics and Evaluation and Research (CBER). *Guidance for Industry Stability Testing of Drug Substances and Drug Products*, June 1998. US Department of Health and Human Services, Food and Drug Administration: Rockville, MD.
- 47 Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973; **52**: 456–467.
- 48 Xiao W et al. Gene therapy vectors based on adeno-associated virus type 1. *J Virol* 1999; **73**: 3994–4003.
- 49 Fisher KJ et al. Recombinant adeno-associated virus for muscle-directed gene therapy. *Nat Med* 1997; **3**: 306–312.
- 50 Nema S, Avis KE. Freeze-thaw studies of a model protein, lactate dehydrogenase, in the presence of cryoprotectants. *J Parent Sci Technol* 1993; **47**: 76–83.
- 51 May JC, Wheeler RM, Etz N, Del Grosso A. Measurement of final container residual moisture in freeze-dried biological products. *Dev Biol Stand* 1992; **74**: 153–164.
- 52 May JC, Grim E, Wheeler RM, West J. Determination of residual moisture content in freeze-dried viral vaccines: Karl Fischer, gravimetric, and thermogravimetric methodologies. *J Biol Stand* 1982; **10**: 249–259.
- 53 Fisher KJ et al. Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J Virol* 1996; **70**: 520–532.
- 54 Croyle MA, Anderson DJ, Roessler BJ, Amidon GL. Development of a highly efficient purification process for recombinant adenoviral vectors for oral gene delivery. *Pharm Dev Technol* 1998; **3**: 365–372.