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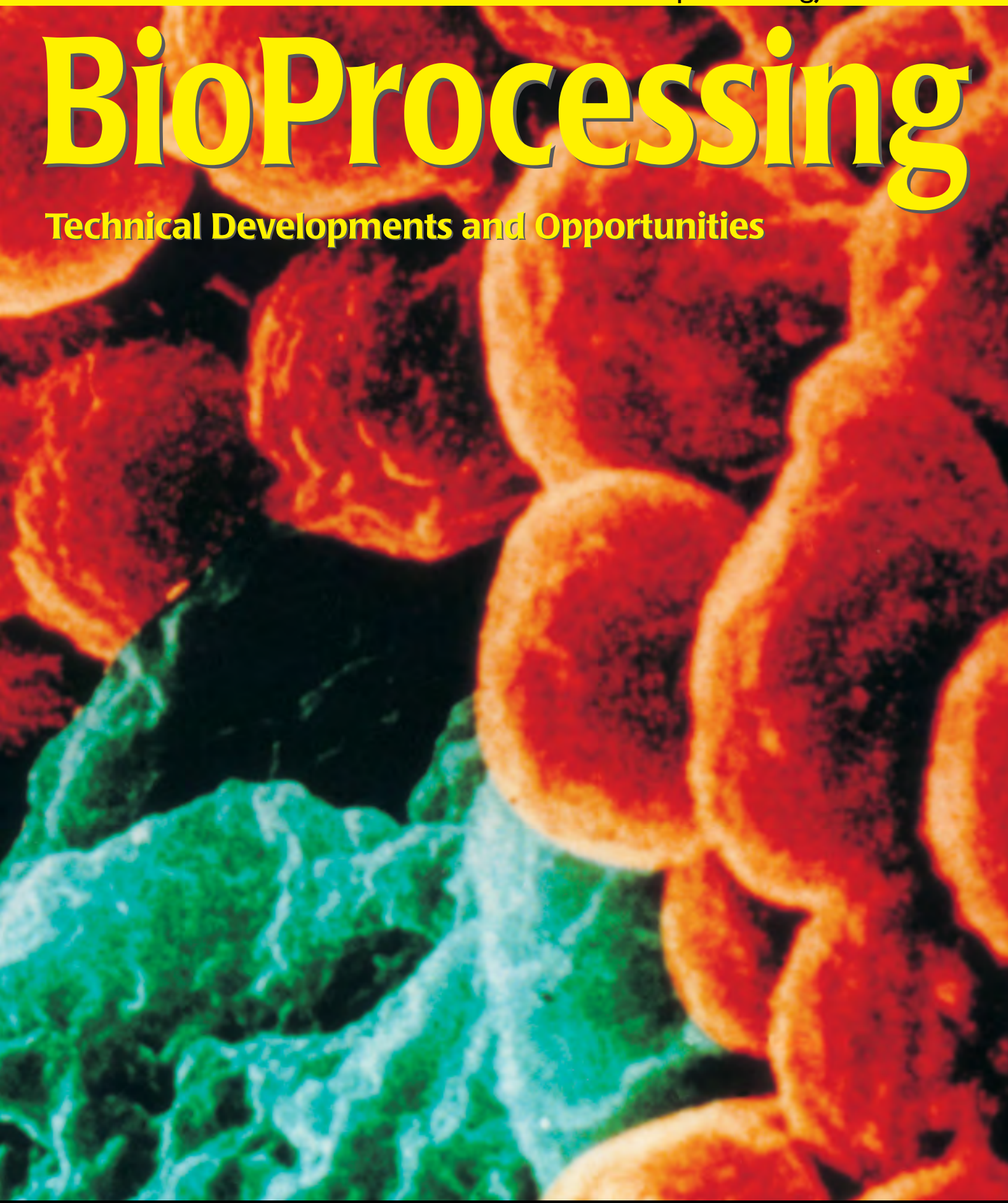

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Development of a Stable Adenoviral Vector Formulation

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A formulation for purified adenoviral vectors was developed that provides stability through freeze-thaw stress and long-term storage at non-frozen temperatures. To evaluate the various test conditions, a panel of stability indicating methods was assembled, which included laser light scattering, HPLC, and transgene expression assays. Preformulation studies were conducted, and the effects of buffer species, pH, cryoprotectants, and salts upon adenoviral vector stability were determined.

The final formulation, GTS, consisted of 2.5% glycerol (w/v) and 25 mM NaCl in 20 mM Tris, pH 8.0. The ability of GTS to confer stability to adenoviral vectors was analyzed by subjecting the vector, AdPDGF-B, to temperature stress, freeze-thaw cycles, and long-term storage.

Stability studies analyzed AdPDGF-B at concentrations up to $1.7x10^{12}$ particles/ml. GTS provided stability through at least 5 freeze-thaw cycles for vector concentrations ranging from $1.7x10^{10}$ to $1.7x10^{12}$ particles/ml. In a 6-month study, GTS afforded stability to adenoviral vector when frozen at -20°C, regardless of the particle concentration. However, at refrigerated temperatures, vector stability was dependent upon particle concentration. At the highest concentration tested ($1.7x10^{12}$ particles/ml), particle disintegration was observed between 3 and 6 months. In contrast, particles at concentrations less than or equal to $1.7x10^{11}$ particles/ml remained stable for at least 6 months at 2-8°C. In a long-term temperature stability study, GTS conferred stability for at least 12 months at -20°C with concentrations ranging from $5.1x10^{10}$ to $5.1x10^{11}$ particles/ml.

These studies were presented to the Adenovirus Reference Material Working Group in March 2001, and resulted in the choice of GTS as the formulation for the Ad5 WT Reference Material.

INTRODUCTION

Typical adenovirus formulations generally provide less than the desired vector stability for gene therapy applications. Historically, formulations have consisted of 10-50% glycerol in phosphate or Tris buffers, with the addition of other excipients such as salts and bovine serum albumin.[1-3] Rigorous formulation development has not been reported until recently.[2,4]

Historical formulations are less than optimal for a number of reasons. First, concentrated glycerol is problematic due to its high viscosity and osmotic pressure. Extensive dilutions are needed to allow accurate mixing and dispensing, and to achieve acceptable tonicity for patient administration. Second, many buffers, such as potassium phosphate, do not maintain their pH upon freezing, which may lead to a rapid loss of protein structure. Third, salt concentrations should be minimized in order to maintain isotonicity. Lastly, for both cost and safety reasons, the absence of serum is now preferred.

Our objective was to develop a formulation that would maintain the stability of purified recombinant adenoviral vectors at clinically relevant temperatures, as well as through freeze-thaw cycles. The desired formulation would exhibit a viscosity that enables accurate dispensing and a tonicity that does not require dilution prior to administration. The formulation development was performed in stages. First, an appropriate panel of stability indicating methods was assembled. Next, a preformulation study was performed that focused on buffer species, pH, cryoprotectants, and salt species. Lastly, a series of freeze-thaw stress studies and long-term stability studies were performed.

The fast, sensitive, and reliable techniques of laser light scattering (LLS) and anion-exchange HPLC (AE-HPLC) were used to obtain physical data. LLS

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Figure 1.
Glycerol (2.5%) provides adenoviral vector stability through a single freeze-thaw cycle.

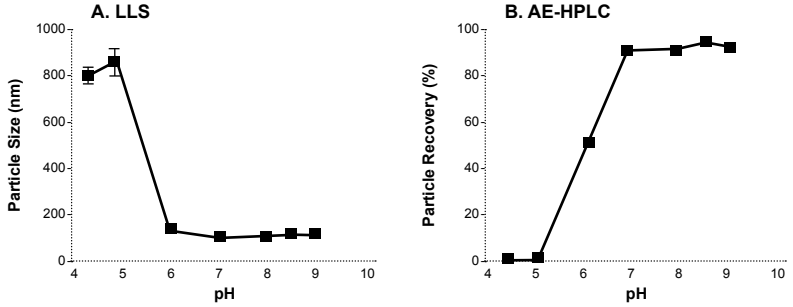

Figure 1. Adenoviral vector maintains stability over a pH range of 7.0 to 9.0. AdPDGF-B was adjusted to $8x10^{10}$ PN/ml in 50 mM sodium phosphate at various pH levels, maintained at 20 to 25°C for 24 hr, and analyzed by (A) LLS and (B) AE-HPLC. Controls were test samples (pH 8.0) maintained at <-65°C.

Table 1.
Glycerol (2.5%) provides adenoviral vector stability through a single freeze-thaw cycle.

Cryoprotectants (w/v)	AdPDGF-B (PN/mL)	Number Freeze-Thaw Cycles	Particle Size (nm)	Polydispersity
Control (no cryoprotectant)	$1.2x0^{12}$	0	101	0.08
		1	>1000	1.00
2.5% Glycerol	$2.0x10^{12}$	0	102	0.10
		1	103	0.11
		3	>1000	1.00
5% Glycerol	$2.1x10^{12}$	0	104	0.04
		1	>1000	1.00
10% Glycerol	$1.6x10^{12}$	0	110	0.09
		1	>1000	1.00
5% Sucrose	$2.0x10^{12}$	0	103	0.07
		1	>1000	1.00

Table 1. Glycerol (2.5%) provides adenoviral vector stability through a single freeze-thaw cycle. AdPDGF-B, at concentrations of $1.2x10^{12}$ to $2.1x10^{12}$ PN/ml, was formulated in 0, 2.5, 5, or 10% glycerol, or 5% sucrose in 20 mM Tris, pH 8.0 and aliquoted in 200 µl volumes. Test samples were then subjected to multiple freeze-thaw cycles and analyzed by LLS for particle integrity.

Table 2.
Salt provides adenoviral vector stability through multiple freeze-thaw cycles.

NaCl (mM)	Number Freeze-Thaw Cycles	Particle Size (nm)	Polydispersity
0	0	103	0.11
	1	125	0.21
	3	>1000	1.00
10	0	106	0.10
	3	112	0.09
	5	105	0.21
25	0	103	0.08
	1	101	0.13
	5	103	0.09
50	0	101	0.03
	1	103	0.09
	5	103	0.06

Table 2. Salt provides adenoviral vector stability through multiple freeze-thaw cycles. AdPDGF-B ($7.0x10^{11}$ PN/ml) was formulated in 0, 10, 25, or 50 mM NaCl and 2.5% glycerol in 20 mM Tris at pH 8.0 and aliquoted in 200 µl volumes. Test samples were then subjected to multiple freeze-thaw cycles and analyzed by LLS for particle integrity.

provided information on particle integrity and the mechanisms of particle destabilization. Particle aggregation was indicated by an increase in particle size and polydispersity, while capsid disintegration was indicated by a decrease in the intensity of scattered light with vectors which have maintained particle size and polydispersity.

AE-HPLC provided information on particle integrity and concentration as derived from the retention time, spectral scan, and peak area.[5,6] To obtain functional data, a transduction ELISA was used. The transduction ELISA measured the levels of transgene-encoded protein (PDGF-B) following adenoviral infection. Taken together, these three methods provided a useful assessment of adenoviral stability.

Preformulation studies identified GTS (2.5% (w/v) glycerol and 25 mM NaCl in 20 mM Tris at pH 8.0) as the formulation of choice for adenoviral vectors. Stress studies then confirmed the ability of GTS to provide adenoviral stability through multiple freeze-thaw cycles and during long-term storage at both refrigerated and clinically relevant storage temperatures.

MATERIALS AND METHODS

Adenoviral vectors. Recombinant adenovirus type 5 (E1- and E3-deleted), encoding either β-galactosidase (Adβ–gal) or platelet-derived growth factor-β (AdPDGF-β), was amplified in the human embryonal kidney packaging cell line, 293 [American Type Culture Collection, Manassas, VA][7]. Adβgal was purified from cell lysates using CsCl-gradients, and then formulated into GTS. AdPDGF-B was purified from cell lysates by Introgen Therapeutics, Inc. (Houston, TX) using anion exchange chromatography, and then formulated into GTS. A single lot was prepared at $1.6x10^{12}$ particles (PN)/ml for use in the preformulation studies. Two additional cGMP lots were prepared for use in the stability studies. Lot 1 was filled into sterile, 1.8 ml cryovials (1.2 ml/vial) at $1.7x10^{10}$, $1.7x10^{11}$ and $1.7x10^{12}$ PN/ml. Lot 2 was filled into sterile, 3 mL flint glass vials (1.25 ml/vial with a butyl stopper and flip-off button crimp) at $5.1x10^{10}$, $1.7x10^{11}$, and $5.1x10^{11}$ PN/ml.

Freeze-thaw Stress. Preformulation test samples in Eppendorf tubes, or AdPDGF-B Lot 1 samples in their original vials, were frozen in an ethanol-dry ice bath (-72°C) for 5 minutes and then thawed at room temperature (RT 20 to 25°C) for 15 to 20 min. AdPDGF-B Lot 2 samples, in their original vials, were frozen in a non-cycling -20°C freezer for >3 hr and thawed at RT (20 to 25°C) for 30 to 60 minutes.

Long-term Temperature Stability. Vials were stored in temperature-controlled environments of: <-65°C (control), -20°C, and 2-8°C. Lot 1 samples were analyzed at the 2 week, 1 month, 3 month, and 6 month time points. Lot 2 samples were analyzed at the 1 month, 3 month, 6 month, and 12 month time points. An 18 month time point is in progress.

Laser Light Scattering (LLS). The average virus particle size was determined by LLS using a Zetasizer 5000 (Malvern Instruments, England). LLS provided three types of data: Particle size, polydispersity, and light intensity. The average hydrodynamic diameter was measured with an argon-ion laser operating at 488 nm, 15 to 50 milliwatts, and a 90° angle. The hydrodynamic diameter described the apparent size of the particle as it existed in a solution, and was reported as the average size of the entire population. Using these conditions, adenoviral vectors were observed to have an apparent size of 80 to 120 nm.

The polydispersity (or homogeneity) of the population described the relative particle size distribution within the population.

The polydispersity was reported using an index ranging from zero to one. As the polydispersity index approached one, the sample was more likely to contain multiple populations of varying size. For purified adenovirus under these conditions, a polydispersity index less than 0.2 indicated a relatively monodispersed distribution. Light intensity was given in kilocounts per second (kcps), and together with size and polydispersity, was used to assess the mechanism of particle loss. The intensity of the light was proportional to the vector concentration.

Anion Exchange-HPLC. AE-HPLC was used to assess the integrity and concentration of adenoviral particles. AE-HPLC utilized a Resource Q column (Amersham-Pharmacia, Uppsala, Sweden) attached to an HP1050 HPLC (Hewlett-Packard, Santa Clarita, CA). Using published methodology,[8,5,6] adenoviral vector was eluted at a retention time of about 8-8.5 minutes with a detection limit of about $2x10^{10}$ PN/ml.

AdPDGF-B Transduction/PDGF-BB ELISA. To confirm whether the AdPDGF-B particles were infective and expressed PDGF-B, test samples were subjected to the transgene expression assay described previously, the AdPDGF-B transduction/PDGF-BB ELISA.[9] Briefly, 293 cells were transduced at multiple concentrations, and 18 hours later, cell lysates and supernatants were subjected to ELISA analysis. The human PDGF receptor β/Fc chimera (R&D Systems, Minneapolis, MN) was used to capture the PDGF-BB dimer. The PDGF-BB protein was then detected using a biotin-labeled PDGF-BB antibody (R&D Systems) with a colorimetric readout. Recombinant human PDGF-BB protein was used as a positive control (Life Technologies, Rockville, MD). Activity was described as ng PDGF-BB/ml at defined PN/cell ratios.

RESULTS

Preformulation Studies

Buffer Species Studies. Numerous buffers were analyzed for their ability to protect adenoviral vectors from freeze-thaw stress (data not shown). Adβgal was prepared at $1.7x10^{11}$ PN/ml in varying concentrations (10 mM to 160 mM) of glycylglycine, sodium phosphate, sodium citrate, Tris-HCl, potassium phosphate, and HEPES buffer at pH 7.4. Formulated test samples were placed into -20°C storage. The following day, samples were thawed at RT and immediately analyzed by LLS.

An increase in particle size was observed with glycylglycine and sodium phosphate buffers at concentrations >20 mM. However, sodium citrate, Tris-HCl, potassium phosphate, and HEPES all maintained particle integrity through the freeze-thaw (F/T) cycle at all concentrations.

Although it appeared reasonable to use any number of buffer species in formulation development, further studies focused upon Tris, as previous reports supported its ability to maintain pH upon freezing.[10,11] In addition, because the pKa of Tris is 7.82 at 37°C and 8.3 at 20°C, it provides good buffering capacity at physiological pH.[11,12] A Tris concentration of 20 mM was selected for adequate buffering capacity, and to limit the contribution of the buffer to the tonicity of the formulation.

pH Studies. The effect of pH on vector stability was determined using 50 mM sodium phosphate. Phosphate buffer was used because of its wide buffering capacity, which permitted analysis over a broad pH range without changing buffer species.

Following the Henderson-Hasselbalch equation, buffers were prepared using both the monobasic and dibasic forms of sodium phosphate, and the pH was confirmed using a pH meter. The research lot of AdPDGF-B was prepared at $8x10^{10}$ PN/ml from pH 4.5 to 9.0 (200 µl/Eppendorf tube). Formulated samples were incubated at RT for 24 hours, and then analyzed using LLS and AE-HPLC (Figure 1). An increase in particle size was observed at pH <6.0, and a loss in particle recovery was observed at pH <7.0. These data indicated that the adenoviral samples were stable over a pH range of 7.0 to 9.0. Preformulation analysis of cryoprotectant and salt effects was performed using 20 mM Tris at pH 8.0.

Cryoprotectant and Salt Studies. Next, we assessed the ability of cryoprotectants to protect AdPDGF-B from freeze-thaw stress. Varying concentrations of glycerol or sucrose were prepared in 20 mM Tris at pH 8.0. AdPDGF-B was admixed with the various formulations (200 µL/Eppendorf tube), subjected to 1-3 cycles of F/T, and analyzed for particle size and integrity by LLS (Table 1

Table 3. GTS affords adenoviral vector stability to freeze-thaw stress.

AdPDGF-B (PN/ml)	Number Freeze-Thaw Cycles	LLS Particle Size (nm)	LLS Polydis-persity	LLS Light Intensity (kcps)	AE-HPLC PN Recovery (Percent)	Transduction ELISA (ng PDGF-BB/ml)
	0	ND	ND	ND	100	1.6 ± 0.39
$1.7x10^{10}$	1	ND	ND	ND	101	2.0 ± 0.40
	3	ND	ND	ND	100	2.6 ± 0.42
	5	ND	ND	ND	104	2.5 ± 0.01
	0	100	0.10	26	100	4.7 ± 0.72
$1.7x10^{11}$	1	102	0.10	25	100	5.0 ± 0.40
	3	100	0.08	24	97	3.4 ± 0.53
	5	101	0.10	26	96	3.8 ± 1.2
	0	106	0.06	97	100	3.2 ± 1.2
$1.7x10^{12}$	1	102	0.12	95	99	2.7 ± 0.24
	3	101	0.12	96	98	3.4 ± 0.74
	5	101	0.11	96	100	3.1 ± 0.22

ND; not determined, below the limit of detection for LLS

Table 3. GTS affords adenoviral vector stability to freeze-thaw stress. AdPDGF-B Lot 1 was formulated in GTS and subjected to 5 cycles of freeze-thaw stress. Controls were samples stored at <-65°C and not subjected to additional F/T cycles. Samples were analyzed by LLS, AE-HPLC, and AdPDGF-B Tranduction/PDGF-BB ELISA using an infection concentration of 31 PN/cell.

Table 4. GTS affords adenoviral vector stability for at least six months in -20°C storage at concentrations up to $1.7x10^{12}$ PN/ml.

AdPDGF-B (PN/ml)	Storage Temperature	LLS Particle Size (nm)	LLS Polydis-persity	LLS Light Intensity (kcps)	AE-HPLC PN Recovery (Percent)	Transduction ELISA (ng PDGF-BB/ml)
	<-65°C	ND	ND	ND	100	8.2 ± 1.4
$1.7x10^{10}$	-20°C	ND	ND	ND	84	7.3 ± 0.72
	2-8°C	ND	ND	ND	ND	9.1 ± 0.39
	<-65°C	104	0.11	60	100	9.1 ± 0.35
$1.7x10^{11}$	-20°C	108	0.15	62	98	12.6 ± 2.2
	2-8°C	108	0.12	74	98	12.6 ± 0.28
	<-65°C	ND	ND	ND	100	8.9 ± 1.4
$1.7x10^{12}$	-20°C	100	0.08	479	104	8.1 ± 0.41
	2-8°C	111	0.12	338	35	4.0 ± 0.96

ND; not determined, below the limit of detection for LLS

Table 4. GTS affords adenoviral vector stability for at least six months in -20°C storage at concentrations up to $1.7x10^{12}$ PN/ml. AdPDGF-B Lot 1 was stored at <-65°C, -20°C and 2-8°C over 6 months and analyzed for particle integrity and functional activity using LLS, AE-HPLC, and AdPDGF-B Tranduction/PDGF-BB ELISA (at 500 PN/cell). Controls were samples stored at <-65°C.

shows a limited dataset).

Glycerol (>5%) or 5% sucrose alone did not prevent virus aggregation beyond 1 F/T cycle, while 2.5% glycerol stabilized the vector for a single F/T cycle, but not 3 F/T cycles. Further formulation development focused on 2.5% (w/v) glycerol in 20 mM Tris at pH 8.0. Salts are known to confer stability to proteins in solution.[13]

A number of salts were therefore monitored for their effects including $MgCl_2$, $CaCl_2$, and NaCl. Table 2 presents the effects of 10-50 mM NaCl and 2.5% (w/v) glycerol in 20 mM Tris at pH 8.0. AdPDGF-B was formulated at $7.0x10^{11}$ PN/ml (200 µl/Eppendorf tube), subjected to 3 F/T cycles, and monitored for particle integrity using LLS (Table 2).

The inclusion of NaCl at >10 mM prevented particle aggregation for up to 5 F/T cycles. Additional experiments demonstrated marginal protection at 10 mM NaCl (data not shown). In order to ensure adequate protection, further formulation development used 25 mM NaCl. The preferred formulation was therefore defined as 2.5 % (w/v) glycerol and 25 mM NaCl in 20 mM Tris at pH 8.0 (GTS).

GTS Formulation Stress Studies

The preformulation studies indicated that GTS afforded stability to adenoviral vectors. Therefore, a series of stress studies were performed with two large-scale cGMP production lots of AdPDGF-β.

Freeze-Thaw Stability. Using both Lots 1 and 2 of AdPDGF-B, we analyzed the ability of GTS to maintain particle integrity and biological activity through F/T stress. Individual vials were subjected to multiple freeze-thaw cycles, and then analyzed by LLS, AE-HPLC and AdPDGF-B transduction/PDGF-BB ELISA. The particle size and polydispersity of samples in cryovials at middle and high concentrations ($1.7x10^{11}$ and $1.7x10^{12}$ PN/ml) or of samples in glass vials at middle and high concentrations ($1.7x10^{11}$ and $5.1x10^{11}$ PN/ml) were analyzed following 1, 3, and 5 F/T cycles. Freshly thawed vials were used as controls (<-65°C). The low particle concentration samples from each lot were not analyzed, since they were below the limit of quantitation of the LLS method.

Lot 1 data is shown in Table 3. Lot 2 data is not shown, since the results were identical to those for Lot 1. No change in particle size or polydispersity was observed for any of the samples. Vector concentration, measured by AE-HPLC, indicated quantitative recovery of intact particles. When compared to a freshly thawed control, no changes in PDGF-BB expression were observed by AdPDGF-B transduction/PDGF-BB ELISA. These data indicate that the GTS formulated adenoviral samples remained stable through at least 5 F/T cycles over a concentration range from $1.7x10^{10}$ to $1.7x10^{12}$ PN/ml.

Long-Term Temperature Stability. The stability of AdPDGF-B Lots 1 and 2

was assessed for up to 6 months and 12 months, respectively, at control (<-65°C), anticipated clinical storage (-20°C), and refrigerated (2-8°C) temperatures.

With AdPDGF-B Lot 1, no loss in vector stability was observed at the 2 week, 1 month, or 3 month time points as determined by LLS, AE-HPLC, and AdPDGF-B transduction/PDGF-BB ELISA (data not shown). As demonstrated by the three stability-indicating methods, at 6 months all samples were stable at the storage temperature of -20°C (Table 4). In addition, the low and middle concentration samples ($1.7x10^{10}$ and $1.7x10^{11}$ PN/ml) were stable for at least 6 months when stored at 2-8°C.

However, the 6 month high concentration sample ($1.7x10^{12}$ PN/ml) that was stored at 2-8 °C, showed an almost 65% decrease in intact particle recovery, and approximately a 55% decrease in biological activity. Particle size remained at approximately 100 nm, while the light scattering intensity decreased almost 30%. This indicates particle loss by degradation, as opposed to aggregation. Taken together, these studies demonstrate that GTS formulated adenovirus was stable at -20°C for at least 6 months at concentrations up to $1.7x10^{12}$ PN/ml. When formulated in GTS and stored under refrigerated conditions, a concentration dependent stability was observed for at least 6 months with concentrations $<1.7x10^{11}$ PN/ml. Lot 2 data was of particular interest, since the vials and formulation were identical to those of the Ad5 WT Reference Material (3 ml, flint glass), plus the concentration was similar ($5.0x10^{10}$ vs. $5.1x10^{10}$ PN/ml). With AdPDGF-B Lot 2, no loss in vector stability was observed at the 1 month, 3 month, or 6 month time points as determined by LLS, AE-HPLC, and AdPDGF-B transduction/PDGF-BB ELISA (data not shown). All samples were stable for at least 12 months when stored at -20°C, as indicated by all three stability indicating methods (Table 5).

As demonstrated by AE-HPLC, low and middle concentration samples ($5.1x10^{10}$ and $1.7x10^{11}$ PN/ml) were also stable in 2-8°C storage. LLS and AE-HPLC data indicated stability of the high concentration sample ($5.1x10^{11}$ PN/ml) in 2-8°C storage, with no more than a 20% decrease in particle recovery after 12 months. Although not statistically significant, the ELISA data suggested a loss in biological activity for both the low and high concentration samples at 2-8°C. The pending 18 month sample should determine if this loss in activity is real. Nonetheless, with adenoviral vector formulated similarly to that of the Ad5 WT Reference Material, it is clear that GTS affords stability at -20°C for at least 12 months and at 2-8°C for at least 6 months.

Table 5. GTS affords 12 month stability of adenoviral vector formulated and filled similarly to that of the Ad5 WT Reference Material.

AdPDGF-B (PN/ml)	Storage Temperature	LLS Particle Size (nm)	LLS Polydis-persity	LLS Light Intensity (kcps)	AE-HPLC PN Recovery (Percent)	Transduction ELISA (ng PDGF-BB/ml)
$5.1x10^{10}$	<-65°C	ND	ND	ND	100	22.0 ± 0.8
	-20°C	ND	ND	ND	100	22.6 ± 2.8
	2-8°C	ND	ND	ND	95	15.6 ± 4.6
$1.7x10^{11}$	<-65°C	108	0.12	36	100	ND
	-20°C	114	0.08	41	100	ND
	2-8°C	113	0.08	41	97	ND
$5.1x10^{12}$	<-65°C	109	0.04	128	100	21.8 ± 9.4
	-20°C	112	0.08	119	101	21.9 ± 6.3
	2-8°C	122	0.11	121	81	16.3 ± 8.7

ND; not determined, LLS samples were below the limit of detection

Table 5. GTS formulation confers stability to adenoviral vector formulated and filled similarly to that of the Ad5 WT Reference Material. AdPDGF-B Lot 2 (in 3 ml flint glass vials) was stored at <-65°C, -20°C and 2-8°C over 12 months and analyzed for particle integrity and functional activity using LLS, AE-HPLC, and AdPDGF-B Tranduction/PDGF-BB ELISA (410 PN/cell). Controls were samples stored at <-65°C.

DISCUSSION

A variety of stability indicating methods were used to assess particle integrity, since no single method provided the sensitivity needed to assess both structural integrity and functional activity. Fast and reliable methods were first used to screen formulations, while more time intensive assays were included for final stability assessment.

Laser light scattering (LLS) provided a fast and reliable method to screen formulations for structural characteristics, and provided information on the mechanism of particle degradation.[14] It produced absolute values and therefore was independent of reference standards. However, a detection limit of about $1x10^{11}$ PN/ml restricted its utility. Aggregation was indicated when large particles (>1000 nm) and a heterogeneous population (polydispersity >0.2) were observed. Disintegration was indicated when particle size was around 100 nm, polydispersity remained constant below 0.2, and the light scattering intensity diminished.

Anion exchange-HPLC (AE-HPLC) was another fast method that provided precise results in relation to a reference standard. AE-HPLC was used to determine the concentration of intact particles, and to confirm test article identity by peak retention time and spectral scan profile. In addition, the peak A260/A280 ratio was used as an indication of vector purity [expected range 1.2 to 1.4].[5]

The AdPDGF-B transduction/PDGF-BB ELISA was a more labor-intensive assay, relative to the analytical methods, and therefore was used only as a final screen of test article stability. This assay was used to confirm biological activity since it required the vector to infect cells as well as express the transgene. The results obtained from these analytical methods (LLS, AE-HPLC, and transduction ELISA) were in close agreement with each other throughout these studies, and their use as stability indicating methods was therefore validated.

The choice of components for the GTS formulation was supported by

both empirical and published data. Tris was selected as the buffering agent primarily for its ability to maintain pH upon freezing. This was in contrast to sodium phosphate buffered solutions, which have been observed to drop from pH 7.5 at 25°C to less than pH 4 at -25°C.[10] In addition, Tris provided good buffering capacity at physiologic pH[11], and has been used in pharmaceutical formulations.[15,16] A pH of 8.0 was chosen due to demonstrated vector stability over the range of pH 7 to 9, in accordance with the findings of Nyberg-Hoffman and Aguilar-Cordova.[17]

In order to protect the virus from the damaging effects of freezing and thawing, a cryoprotectant was required. Glycerol concentrations (0-10%), as well as sucrose, were assessed since both are commonly used as pharmaceutical excipients.[15,17,18] Because glycerol has been used in many adenoviral vector production and purification processes, it was deemed more reasonable for this formulation.

The goal was to minimize viscosity while still achieving isotonicity. Therefore, we sought to reduce the glycerol concentration to the minimum amount required for a protective effect. The osmolality of 2.5% glycerol was within the physiological range of ca. 280-300 mOsmol/kg (data not shown), and because the 2.5% formulation provided stability, it was chosen as the final cryoprotectant concentration. Addition of a salt was also found to be required for maintaining vector stability. While as little as 10 mM salt provided a significant stabilizing effect, experimental variation indicated that a slightly higher concentration (25 mM) was warranted in the final formulation.

In summary, an adenoviral vector formulation consisting of 2.5% (w/v) glycerol and 25 mM NaCl in 20 mM Tris at pH 8.0 (GTS) was developed and demonstrated to protect vectors against freeze-thaw and long-term thermal stress. This formulation did not require dilution to achieve physiologically acceptable tonicity prior to administration. Its low viscosity made it easy to dispense, and allowed for precise volume measurement and transfer. GTS maintained adenoviral vector stability through multiple freeze-thaw cycles.

In addition, long-term storage stability was demonstrated at -20°C for vector samples at concentrations up to $1.7x10^{12}$ PN/ml. At refrigerated temperatures of 2-8°C, vector stability was dependent on particle concentration. At high concentrations ($1.7x10^{12}$ PN/ml), particle disintegration was observed between 3 and 6 months. At lower concentrations ($<5.1x10^{11}$ PN/ml), adenovirus remained stable in refrigerated storage for at least 6 months.

It is important that adenoviral vectors be able to withstand common field use conditions such as multiple freeze-thaw cycles and exposure to varying temperatures. Using recommended storage conditions, our studies indicate that the GTS formulation will ensure an adenoviral vector, notably the Ad5 WT Reference Material, will maintain its integrity and activity throughout a period of time typically associated with research and clinical evaluation of gene therapy products.

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