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Investigation on formulation and preparation of adenovirus encoding human endostatin lyophilized powders

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ABSTRACT

A recombinant adenovirus encoding human endostatin gene, E10A, has finished phase II trials for head and neck cancer. However, the rigid storage temperature (−80 °C) and the toxicity of glycerol in the E10A liquid preparation limited its clinical application. In this study, lyophilization was applied to develop a stable E10A lyophilized powder without glycerol that is able to maintain biological activity at 4 °C and suitable for intravenous administration. The E10A lyophilized formulations composed of nontoxic and already clinically used excipients were characterized in terms of the pH change during freezing, the eutectic melting temperature (T_{eu}) and the collapse temperature (T_c). Freeze thawing tests were carried out to examine the protective effect of various excipients during freezing. Mannitol and its combinations with sucrose or inulin showed effective protection of E10A. The E10A lyophilized powders were analyzed by particle size measurement, residual humidity quantification, infectivity assay and gene expression level. An optimized formulation (formulation I1) yielded a good recovery of 76% of the starting infectivity after lyophilization and 89% of the original infectivity after storage at 4 °C for 180 days. Also the gene expression capability of E10A in formulation I1 was maintained after lyophilization. In addition, it was found that the matrix of amorphous excipients, mannitol combinations with sucrose or inulin, was indispensible in protecting E10A against the stress of freezing and dehydration. Hereby, the E10A lyophilized powder with eliminated glycerol toxicity and improved stability could enhance the applicability of E10A for cancer gene therapy through intravenous administration.

1. Introduction

Angiogenesis plays a critical role in cancer development and endostatin is considered one of the most effective inhibitors of angiogenesis found to date (Folkman, 2006; Huang et al., 2007). E10A, a recombinant type 5 adenovirus (Ad5), employs the adenovirus-mediated delivery of human endostatin gene to inhibit tumor growth by antiangiogenesis (Li et al., 2006, 2008). The phase II clinical trial has demonstrated effectiveness of E10A combined with chemotherapy in the treatment of patients with head and neck cancer (ClinicalTrials.gov identifier, NCT00634595).

Preservation of E10A for clinical use has become a significant issue as clinical trials are progressing to phase III and, ultimately, regulatory approval. Presently, E10A preparations for clinical application consisted of glycerol (10%, v/v) in Tris buffers and are stored at −80 °C to ensure biological activity. Apparently, this ultra-low temperature storage is not only very expensive, but causes serious inconvenience to storage and transportation. The liquid formulations also require extensive dilutions in clinical application to reduce the toxicity of the glycerol, which limits the usage of E10A in intravenous administration. Thus, it seems necessary to develop a stable E10A formulation with preservation of biological activity at 4 °C and suitable for intravenous administration.

Lyophilization is a common procedure to alleviate this problem (Rexroad et al., 2002). A typical lyophilization cycle consists of initial freezing, primary drying, and secondary drying stages (Nail et al., 2002). Usually cryo- and lyoprotectants including polyols and sugars have to be employed to protect the viruses from freezing and desiccation stresses. Several studies reported their efforts to develop stable Ad5 formulations. Croyle et al. (1998, 2001) investigated factors including buffer systems, freezing rates, and the protective efficiency of cryoprotectants on the stability of Ad5 during freeze drying process and storage conditions. Their work identified a lyophilized formulation in sucrose showed negligible

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loss of titer for 1 year at 4 °C. Evans et al. (2004) disclosed a stable liquid Ad5 based vaccine formulation that lost <0.1 logs of infectivity after 24 months of storage at 4 °C. These studies presented a comprehensive understanding of the influences of various parameters on the freeze drying process and promoted the application of the lyophilization technique in adenovirus drying.

In this study, we investigated the formulation and preparation of E10A lyophilized powder. The excipients present in our formulation were mainly selected from Generally Recognized As Safe (GRAS) compounds to provide the potential of intravenous administration. Firstly, the E10A formulations were characterized in terms of the pH change during freezing, the eutectic melting temperature (T_{eu}) and the collapse temperature (T_c). Secondly, various formulations were screened in initial freeze thawing tests by analysis of particle size measurement and infectivity assay. Finally, the selected formulations were applied for lyophilization. The lyophilized viruses were evaluated through particle size measurement, infectivity assay, gene expression, residual humidity quantification and storage stability.

2. Materials and methods

2.1. Chemicals and viruses

D-Mannitol, sucrose, magnesium chloride ($MgCl_2$), sodium chloride (NaCl) and Tris (hydroxymethy1)-aminomethane (Tris) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Inulin was obtained from Hefei Bomei Biotechnology Co. Ltd. (Hefei, China). Universal pH indicator was purchased from Keychange Co. Ltd. (Shenzhen, China). All other chemicals were of analytical reagent grade.

The replication-defective recombinant adenoviral vectors carrying the human endostatin gene (E10A) and firefly luciferase gene (Ad/Luc), manufactured and characterized as previously described (Huang et al., 2007; Liang et al., 2007), were provided by Guangzhou Doublle Bioproducts Co. Ltd. (Guangzhou, China). The particle number was 1×10^{12} vp/mL measured by UV spectrophotometric analysis at 260 nm. Purified adenovirus was kept in a storage buffer (10 mM Tris–HCl, pH 8.2, 1 mM $MgCl_2$, 200 mM NaCl) and stored at −80 °C until use.

2.2. Cell culture

The adenovirus transformed human embryonic kidney cell line 293, pulmonary adenocarcinoma cell line A549 and nasopharyngeal carcinoma cell line CNE-1 were kindly provided by the Cancer Center of Sun Yat-sen University (Guangzhou, China). These cells were grown in a Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C and 5% CO_2.

2.3. Sample preparation

E10A or Ad/Luc samples for each formulation, composed according to Table 1, were prepared by mixing thawed adenovirus stock with excipient solutions. Briefly, virus (1×10^{12} vp/mL) was thawed at 4 °C and diluted with sterilized excipient solutions into the desired formulation as listed in Table 1. The final virus concentration ranged from 1×10^9 to 1×10^{11} vp/mL. The initial pH of all preparations was adjusted to 8.2 with 0.1 mol/L NaOH or 0.1 mol/L HCl. After preparation, aliquots of 1 mL were transferred into 3 mL glass vials for freeze thawing and freeze drying.

2.4. Characterizations of the E10A based formulations

2.4.1. pH studies

Samples (1 mL, pH 8.2) for each formulation prepared as described above were mixed with universal pH indicator (10 μL) as previously reported (Eriksson et al., 2003). Samples were frozen for 24 h to −20 and −80 °C, respectively. pH of the frozen products were determined visually and noted for each formulation.

2.4.2. Freeze drying microscopy (FDM)

The eutectic melting temperature (T_{eu}) and collapse temperature (T_c) of different formulations were determined by a freeze drying microscope (Linkam, England) as described previously (Nail et al., 1994). The equipment consists of a small freeze drying chamber containing a temperature-controlled stage, a vacuum pump to ensure the evacuation and an optical window through which the drying sample can be observed by a microscope.

2.5. Freeze thawing tests

Three milliliter flat-bottom screw-cap glass vials filled with 1 mL of virus suspension were used in all experiments. Two cooling procedures were compared: ultra-rapid freezing by immersion into liquid nitrogen (−196 °C for 1 min), slow freezing via placement on a −20 °C refrigerator for 0.5 h and then a −80 °C refrigerator for 5 h. The frozen preparations were kept at 4 °C for thawing. The above freeze thawing cycles were repeated for three times. Different factors have been studied throughout this study, such as: type of cryoprotectant, cryoprotectant concentration and rate of cooling. Subsequently, the samples were analyzed via Dynamic Laser Scanning (DLS) and infectivity assay. Purified adenovirus stocks stored at −80 °C were used as controls.

2.6. Lyophilization of E10A formulations

The lyophilization was carried out in a LYO-0.2 freeze drier (Shanghai Tofflon Science and Technology Co. Ltd., China). Samples prepared as described in Section 2.3 were placed on shelf plates. The freeze drying protocol was the following: (1) freezing, −50 °C for 3 h with a cooling rate of 1 °C/min; (2) primary drying, −50 °C for 60 h at 3 Pa; and (3) secondary drying, 0 °C for 4 h and 20 °C for 8 h at 3 Pa. At the end of the process, the vials were rapidly stoppered under vaccum and stored in desiccators containing dried silica gel at 4 or 37 °C for the stability study. Subsequently, lyophilized powders were assessed with Karl Fisher titration and X-ray diffraction (XRD). Freeze dried samples were reconstituted with 1 mL sterile water for injection and analyzed via DLS, infectivity assay and in vitro transgene expression. Purified adenovirus stocks stored at −80 °C were used as control groups.

2.7. Dynamic laser scanning

The average hydrodynamic diameter and the polydispersity of viruses were determined via DLS using a Malvern Instrument Zetasizer Nano-S with scattering angle of 173° and 632-nm red laser at 25 °C. The final to initial size ratio (S_F/S_I) was also calculated. All the analyses were run in triplicate.

2.8. Karl Fisher titration

The residual humidity quantification in all freeze dried preparations was carried out by a Karl Fisher titration (Metler Toledo titrator DL38, Suisse) using methanol as a sample solvent.

Table 1
Compositions of the E10 lyophilized powder formulations.

Code	Tris (mM)	NaCl (mM)	$MgCl_2$ (mM)	Mannitol % (w/v)	Sucrose % (w/v)	Inulin % (w/v)
C	10	100	1	–	–	–
M	10	100	1	5	–	–
S1	10	100	1	5	5	–
S2	10	100	1	5	10	–
I1	10	100	1	5	–	5
I2	10	100	1	5	–	10

2.9. X-ray diffraction (XRD)

Sample crystallization was studied by a X-ray diffractometer (D/max 2200, Rigaku, Japan). The scans were conducted in the 2θ range from 5° to 45° at the rate of 6°/min.

2.10. Infectivity assay

Infectivity assay was used to gauge the amount of live virus in preparations. HEK 293 cells, permissive to replication of adenovirus, were seeded on 24-well plates at a density of 2.5×10^5 cells/well. Adenovirus samples 10-fold serially diluted in serum free medium were applied to the 293 cells and incubated at 37 °C, 5% CO_2 for 48 h. The medium was aspirated, and viral titers in infectious units (ifu/mL) were determined using the BD Adeno-X-Rapid Titer kit (BD Biosciences Clontech) according to the manufacturer's protocols.

2.11. In vitro transgene expression

2.11.1. Luciferase activity assay

A549 cells were seeded in 24-well plates at an initial density of 2×10^5 cells/well. After 24 h incubation, adenovirus samples containing the luciferase gene were added to the 24-well plates at increasing multiplicity of infection (MOI). The transduced cells were incubated for 3 h, and then medium containing virus was replaced with fresh medium containing serum and incubated for an additional 24 h. The luciferase assay was performed according to the manufacturer's protocols (Promega E1500, USA) on a Sirius luminometer (Berthold Detection Systems, Germany). All data are expressed as relative light units (RLU).

2.11.2. Endostatin protein expression

The supernatants of CNE-1 cells after 72 h of infection with E10A at MOI of 10 were harvested and human endostatin protein was quantitated by human endostatin enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., USA), according to the manufacturer's instructions.

2.12. Storage stability of E10A

Sealed vials of freeze dried E10A were stored in desiccators containing dried silica gel at 4 or 37 °C. At predetermined time intervals, the infectivity of rehydrated E10A was measured to evaluate the virus stability.

2.13. Statistical analysis

Data are presented as mean values ± standard deviation (SD). Statistical tests were performed with the Student's t test. All statistical tests were two-tailed tests and a value of $p < 0.05$ was considered significant and specified in figures with asterisks.

3. Results and discussion

3.1. Formulations and characteristics of E10A lyophilized powders

3.1.1. Compositions of E10A formulations

The compositions of E10A formulations are listed in Table 1. The virus concentration ranged from 1×10^9 to 1×10^{11} vp/mL. All formulations were adjusted to pH 8.2 in Tris buffer to prevent the degradation of E10A in aqueous solution during the lyophilization based on our previous work (data not shown) suggesting that the E10A solution was stable in Tris buffer at pH 8.2.

The excipients present in our formulations were mainly selected from GRAS list to provide the potential of intravenous administration. The base formulation C contained 10 mM Tris, 100 mM NaCl and 1 mM $MgCl_2$. Furthermore, other formulation candidates (M, S1, S2, I1, I2) were added into different concentrations of protectants (Table 1). To achieve the lyophilized virus product with both pharmaceutical accepted appearance and maintained virus bioactivity, a typical formulation mainly consists of a buffering agent, a bulking agent and a virus protectant (Wang, 2000). In our study, Tris was selected as the buffer. Mannitol is a common bulking agent for lyophilization due to its excellent cake-forming property (Johnson et al., 2002). Also it could stabilize viruses to some extent. Sucrose and inulin have been reported as protectants in virus lyophilized formulations (Croyle et al., 2001; De Jonge et al., 2007; Geeraedts et al., 2010) and thus they were chosen as protectants for E10A in our formulation. The composition design of the E10 lyophilized powder formulations took advantage of the cake-forming property of mannitol and virus protection of sucrose or inulin to offer the lyophilized sample a good appearance and a relatively small changed bioactivity. In comparison with the currently applied E10A liquid formulation (10 mM Tris, 100 mM NaCl, 1 mM $MgCl_2$ and 10%, v/v glycerol), our newly formulations avoided the use of glycerol and is possible of intravenous administration.

3.1.2. The pH shift for formulations during freezing

The objective of the pH test was to evaluate the effect of formulation on solution pH during freezing. Our previous work demonstrated the stability of E10A liquid preparation within the pH range of 7.0–8.2 over a 14-day period at 4 °C (data not shown). Furthermore, Ad5 preparations were quite sensitive to pH changes upon freezing (Croyle et al., 2001). Certain components of the adenoviral capsid are unstable at low pH in a frozen state. The penton base which is responsible for holding the viral fiber in place would be discharged from the adenoviral capsid at a pH below 5.0. This would compromise the virus ability of attachment and entry to cells (Stewart and Burnett, 1995).

Table 2 lists the results of pH changes of six different formulations during freezing. All formulations except formulations C and M maintained original pH upon freezing at −20 °C and −80 °C. The pH of formulation C with no protectants present dropped by 1.5 units. Formulation M with only mannitol as cryoprotectant dropped by 0.5 pH units. It is likely that solutes including mannitol, sucrose and inulin inhibited crystallizaion of buffer species, leading to smaller pH shifs upon freezing (Eriksson et al., 2003). These results revealed that mannitol plus sucrose and mannitol plus inulin both act as

Table 2
Characteristics of the E10A lyophilized powder formulations.

Formulation	pH shift during freezing[a]		T_{eu} (°C)	T_c (°C)
	−20 °C	−80 °C		
C	−1.5	−1.5	–	−50
M	−0.5	−0.5	−12	−45
S1	0	0	−13	−36
S2	0	0	−13	−39
I1	0	0	−12	−37
I2	0	0	−14	−38

[a] The pH of all formulations before freezing was 8.2.

more effective pH stabilizers of Tris buffer than single mannitol during freezing.

3.1.3. Characteristic temperatures for formulations

Rational design of the lyophilization cycle is based on the evaluation of critical formulation temperatures including T_{eu} and T_c, where T_{eu} is the eutectic melting temperature of the frozen formulaiton and T_c is the collapse temperature of the given formulation (Nail et al., 2002).

Table 2 displays the T_{eu} and T_c for each formulation. Formulation I2 exhibited the lowest T_{eu} −14 °C and formulation C showed the lowest T_c −50 °C of all samples. It is well known that the shelf temperature should be controlled below T_{eu} of the formulation to guarantee the total solidification of a frozen sample during freezing and below T_c during primary drying to maintain product elegance. If the product temperature during primary drying exceeds T_c, the material will undergo vicious flow, resulting in shrinkage or collapse within the product matrix (Meister and Gieseler, 2009). Based on above information, the average product temperature during the freezing and primary drying step under our conditions was about −50 °C not exceeding the T_{eu} and T_c of all formulations.

3.2. Freeze thawing

Freezing is a critical step for the virus lyophilization. The adenovirus activity can be influenced by exposure to ice–water interfaces, salt and virus concentration effects, pH shifts due to selective crystallization of buffer species and mechanical damage by growing ice crystals (Rexroad et al., 2002).

In fact, as shown in Table 3 and Fig. 1, formulation C lacking protectants, had the largest S_F/S_I (1.71 during fast freezing and 1.33 during slow freezing) and lost about 0.96 logs (89%) of infectivity after freeze thawing cycles indicating adenovirus could not undertake the stress without protection. It could be assumed that the crystallization of ice and cryo-concentrated solution may exercise a mechanical stress on viruses resulting in their destabilization. Thus, various cryoprotectants have to be added to stabilize the therapeutics during freezing (Abdelwahed et al., 2006). According to vitrification hypotheses, cryoprotectant solution becomes freeze-concentrated and then forms a stable glass during freezing. The glass matrix trapped the lyophilized therapeutics to prevent their aggregation and protect them against the mechanical stress of ice crystals (Chen et al., 2010).

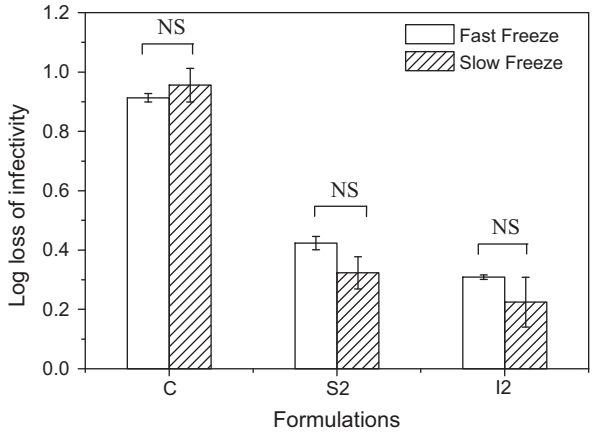

Fig. 1. Effect of freezing rate on the E10A infectivity during freeze thawing cycles. Loss of infectivity was determined by comparison to a −80 °C control for each sample. NS, not significant ($p > 0.1$).

The purpose of freeze thawing tests was to examine the effectiveness of cryoprotectants in stabilizing E10A against freezing stress with regard to prevention of E10A aggregation and preservation of the infectivity. Two cooling methods were employed. Fast freezing was obtained by immersing the samples into liquid nitrogen for 1 min and slow freezing was achieved by placement on a −20 °C refrigerator for 0.5 h and then a −80 °C refrigerator for 5 h.

3.2.1. Effect of freeze thawing on E10A sizes

The sizes of E10A before and after freeze thawing are presented in Table 3. In formulation C, a significant increase in size leading to the largest S_F/S_I 1.71 was observed when a fast freezing procedure was applied, presumably because small ice crystals formed and the relative area of ice–liquid interface where viruses tend to be inactive is larger after rapid freezing (Chang et al., 1996). However, freezing rate did not significantly affect the virus size in formulations with cryoprotectants upon freeze thawing. Cui et al. (2003) also reported the similar results that the rate of freezing had a negligible effect on the particle size of the freeze thawed mannan-coated nanoparticles with cryoprotectants.

Looking at Table 3 in more detail it is found that for the tested E10A formulations mannitol plus sucrose (formulations S1 and S2) and mannitol plus inulin (formulations I1 and I2) preserved the adenovirus sizes in a better fashion than single mannitol (formulation M) during slow freezing. According to the result of polydispersity (Table 3), no serious aggregation of viruses in all formulations occurred through freeze thawing cycles. The polydispersity in formulations I1 and I2 could be attributed to the presence of both E10A and inulin, because the inulin in Formulations I1 and I2 is a biopolymer, which can self-aggregate to form nanoparticles in aqueous solution and then lead to a larger polydispersity. However, the peak of E10A in Formulation I1 or I2 was single. The phenomena of inulin-aggregation and increased polydispersity have been reported by Dan et al. (2009).

3.2.2. Effect of freeze thawing on E10A infectivity

The log loss of adenovirus infectivity after freeze thawing are shown in Figs. 1 and 2. Generally, cryoprotectants addition significantly increased the recovery of the viral infectivity after freeze thawing cycles. Formulations S2 and I2 lost more or less 0.3 logs (50%) of infectivity, independently from the freezing speed after freeze thawing cycles (Fig. 1). Mannitol plus sucrose (formulations S1 and S2) and mannitol plus inulin (formulations I1 and I2) have comparable protection of adenovirus (Fig. 2). Mannitol combinations exhibit better protection capabilities than single mannitol (formulation M) which showed 0.60 logs loss (75%) of infectivity (Fig. 2). In addition, it was found that increasing the concentration of sucrose or inulin from 5% to 10% (w/v) did not improve the virus stability significantly (Fig. 2), suggesting 5% mannitol plus 5% sucrose or 5% mannitol plus 5% inulin were enough to maintain the adenovirus infectivity during freeze thawing cycles.

The infectivity and the size polydispersity of E10A before and after freeze thawing were studied and the results are displayed in Table 3, Figs. 1 and 2. In general, the size polydispersity of E10A increased after freeze thawing and the infectivity exhibited a

Table 3
Sizes of E10A before and after freeze thawing.

Formulation	Freezing rate	Hydrodynamic diameter (nm)		Polydispersity		S_F/S_I
		Before freeze thawing	After freeze thawing	Before freeze thawing	After freeze thawing	
C	Fast freeze	126.5 ± 2.1	216.0 ± 18.4	0.172	0.234	1.71
S2		126.6 ± 1.1	133.5 ± 10.7	0.156	0.307	1.05
I2		124.6 ± 2.3	118.3 ± 2.2	0.672	0.609	0.95
C	Slow freeze	126.5 ± 2.1	168.8 ± 8.6	0.172	0.290	1.33
M		129.0 ± 2.8	144.5 ± 13.6	0.126	0.227	1.12
S1		131.1 ± 5.8	126.5 ± 1.8	0.197	0.396	0.97
S2		126.6 ± 1.1	134.2 ± 11.0	0.156	0.352	1.06
I1		126.8 ± 3.0	118.7 ± 7.5	0.577	0.555	0.94
I2		124.6 ± 2.3	108.2 ± 2.4	0.672	0.594	0.87

corresponding decrease. This result is consistent with previous reports (Obenauer-Kutner et al., 2002; Renteria et al., 2010).

In conclusion of the promising results in terms of E10A size and infectivity obtained from formulations containing mannitol and its combinations with sucrose or inulin, these formulations were considered for the further lyophilization studies. Also formulation C without protectants was chosen as further control to confirm the results obtained from freeze thawing tests.

3.3. Lyophilization

The lyophilization of different formulations was carried out based on the assessment of formulations characteristic temperatures and freeze thawing tests. After freeze drying, all lyophilized powders showed elegant appearance, desirable reconstitution time within 30 s by visually observation, and residual moisture content below 2.0% by Karl Fisher titration. All reconstituted formulations were analyzed in terms of size and infectivity.

3.3.1. Effect of lyophilization on E10A size

The results of E10A sizes in formulations before and after lyophilization are presented in Table 4. In general, after lyophilization the mean hydrodynamic diameters of the adenovirus increased. The highest size increase was observed in formulation C, for which lyophilization process caused serious aggregation non-measurable by DLS. Formulations S1 and S2 had a slightly larger S_F/S_I of around 2.0. Formulations I1 and I2 presented the best protection, for which S_F/S_I was close to 1.0.

3.3.2. Effect of lyophilization on E10A infectivity

Fig. 3 shows the log loss of E10A infectivity after lyophilization. The protective properties of formulations except formulation M during freeze drying are similar to those in freeze thawing. E10A in formulations C and M were almost completely deprived the infectivity (>2.0 log loss). Formulations S1 and S2 preserved approximately 56% of the original infectivity (0.25 log loss). Formulations I1 and I2 exhibited the best protection, for which about 76% of the starting infectivity (0.12 log loss) was maintained. The effect of lyophilization on the infectivity and the size polydispersity of E10A (Table 4 and Fig. 3) was similar to that of freeze thawing. Generally, as the size polydispersity after lyophilization increased, the infectivity of E10A decreased.

Considering the lyophilized powder properties, reconstitution time, moisture content, E10A sizes and infectivity, formulation I1 (5% mannitol plus 5% inulin) efficiently protected adenovirus formulations during the lyophilization. As formulations S2 and I2 display no significant difference concerning the infectivity compared to S1 and I1, respectively, they were not chosen for further assessment.

3.3.3. Effect of protectants crystallization on E10A infectivity

It is worth noting that formulation M exhibits far better protection of E10A infectivity during freeze thawing cycles than that in lyophilization (Figs. 2 and 3). The different protective properties of formulation M were largely attributed to the crystallization behaviors of the cryoprotectants in freeze drying process. The protectants in amorphous state seemed more effective than crystal ones in protecting E10A against stresses during freezing and dehydration steps.

Crystallization is considered to deprive excipents of the molecular interactions such as hydrogen bonding with proteins that are necessary to maintain the native protein structure against stress (Abdelwahed et al., 2006). It has been found that some co-solutes such as NaCl and sucrose can inhibit mannitol crystallization. At a

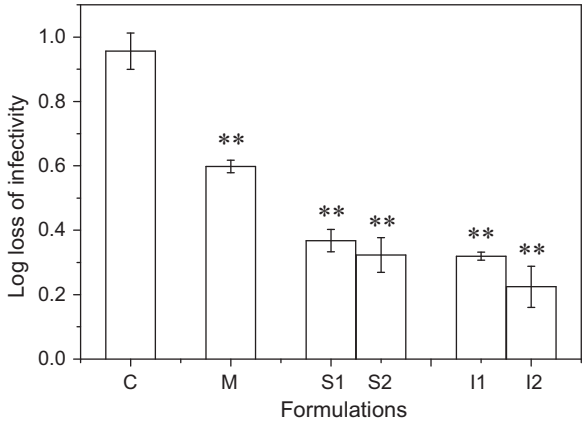

Fig. 2. Effect of formulation on the E10A infectivity during freeze thawing cycles. Loss of infectivity was determined by comparison to a −80 °C control for each sample. *$p < 0.05$ and **$p < 0.01$ vs. formulation C.

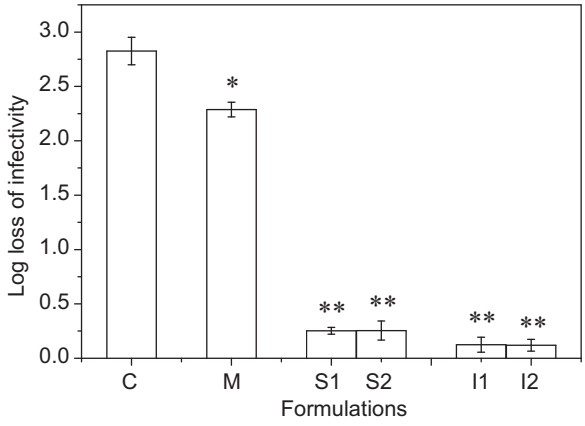

Fig. 3. Effect of lyophilization on the E10A infectivity during freeze drying. Loss of infectivity was determined by comparison to a −80 °C control for each sample. *$p < 0.05$ and **$p < 0.01$ vs. formulation C.

Table 4
Sizes of E10A before and after lyophilization.

Formulation	Hydrodynamic diameter (nm)		Polydispersity		S_F/S_I
	Before lyophilization	After lyophilization	Before lyophilization	After lyophilization	
C	126.5 ± 2.1	Aggregate	0.172	Aggregate	–
M	129.0 ± 2.8	370.1 ± 11.3	0.126	0.374	2.94
S1	131.1 ± 5.8	241.7 ± 70.5	0.197	0.381	1.91
S2	126.6 ± 1.1	262.5 ± 33.8	0.156	0.375	2.08
I1	126.8 ± 3.0	146.2 ± 12.1	0.577	0.616	1.16
I2	124.6 ± 2.3	138.3 ± 8.5	0.672	0.662	1.10

concentration of 0.5% (w/v), NaCl significantly inhibited mannitol crystallization upon freezing (Telang et al., 2003). Each formulation in our study contained 100 mM (0.585%, w/v) NaCl, and therefore mannitol remained in amorphous phase upon cooling which accounted for protection capability of formulation M during freezing stage. The inhibitory effect of NaCl has been explained by the slight melt miscibility of mannitol and NaCl which may provide cohesive force to inhibit crystallization (Telang et al., 2003).

By contrast, lyophilization of mannitol in the presence of NaCl will not generate any significant amount of amorphous mannitol, since the removal of water from the freeze-concentrate will eventually induce both NaCl and mannitol to crystallize (Telang et al., 2003). The crystallization of mannitol could interpret the loss of protection for E10A in formulation M. To produce amorphous mannitol by freeze drying, a second additive such as sucrose could be used. In our formulations, sucrose and inulin was chosen as the second additive respectively because of their nontoxicity and already clinically application. The ratio of mannitol to sucrose or mannitol to inulin was fixed to 1:1 (formulations S1 and I1) and 1:2 (formulations S2 and I2) in order to retain excipients amorphous during all the stages of freeze drying. X-ray diffraction analysis was conducted to examine the physical states of lyophilized powders.

X-ray diffraction demonstrated that the pure mannitol or sucrose powder before and after lyophilization was crystalline (Fig. 4A). The inulin was in amorphous state before and after lyophilization (Fig. 4B). In the XRD patterns of freeze dried formulation S1 and formulation I1, no typical and distinct peaks (the diffraction patterns were analyzed referring to JCPDS-database) for mannitol or sucrose were detected compared to physical mixtures, indicating that the crystallization process was inhibited. Consequently, the amorphous phase existed as a hard and brittle material, and large-scale molecular movement was inhibited,

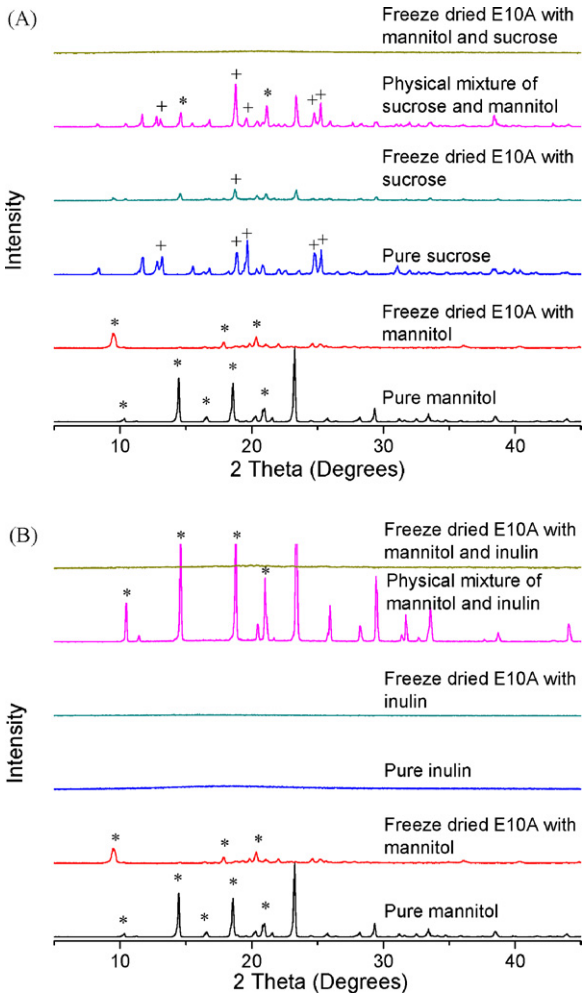

Fig. 4. X-ray diffraction patterns of formulation S1 (A) and I1 (B) before and after lyophilization. The peaks attributed to mannitol and sucrose are indicated by * and +, respectively.

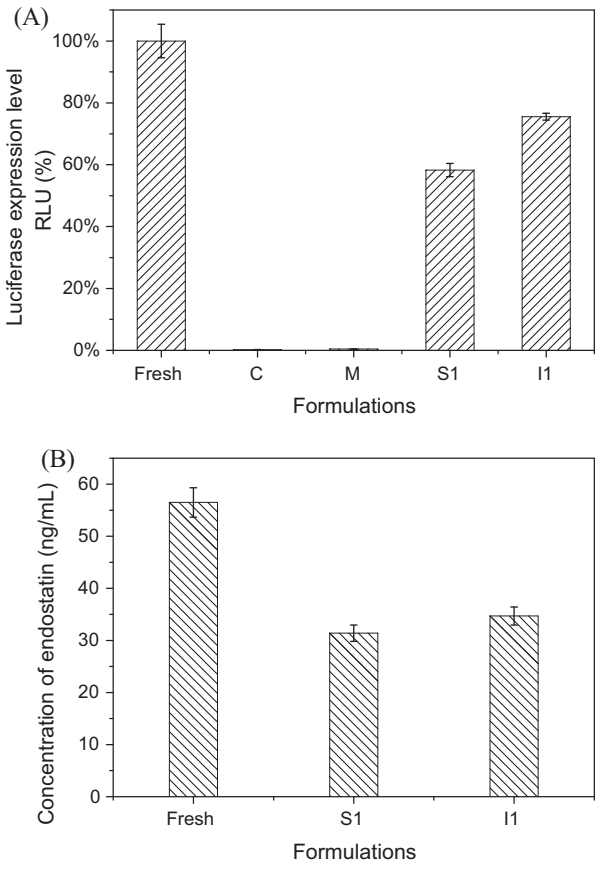

Fig. 5. Transgene expression of Ad/Luc or E10A after lyophilization. (A) Luciferase expression. (B) Endostatin protein expression. The luciferase activity of freshly purified adenovirus stock was set as 100% (mean ± SD, $n = 3$).

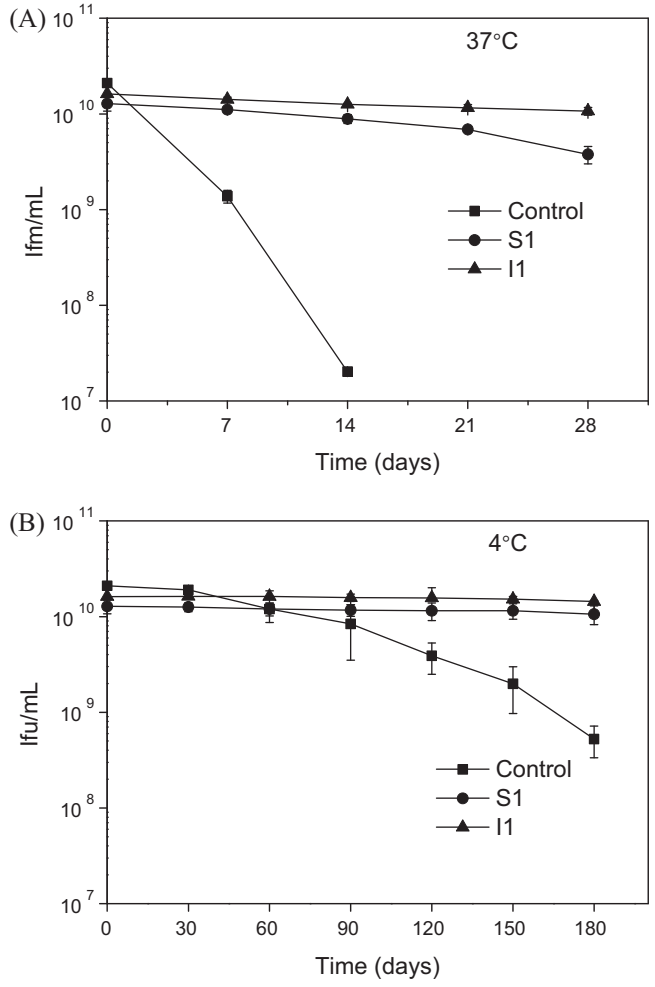

Fig. 6. Storage stability of E10A lyophilized powders. E10A stock solution (squares), freeze dried in formulations S1 (circles), I1 (triangles) were stored at 37 °C (A) and 4 °C (B). At the indicated time points, the infectivity was assayed. Each point together with error bar represents the mean ± SD ($n = 3$).

achieving effective preservation of E10A. Formulation S2 (5% mannitol plus 10% sucrose) and formulation I2 (5% mannitol plus 10% inulin) showed identical XRD patterns to formulaiton S1 (5% mannitol plus 5% sucrose) and I1 (5% mannitol plus 5% inulin) respectively (data not shown), suggesting crystallization was also inhibited in these formulations. Lueckel et al. (1998) pointed that mannitol would not crystallize if its concentration was equal or lower than the sucrose concentration. These are consistent with our results. Manno et al. (2009) reported the addition of inulin to durum wheat pasta lowered the crystallinity of pasta. In our test, we proved inulin could inhibit the crystallization of mannitol.

3.4. In vitro transgene expression of E10A lyophilized powder

A further parameter for the integrity and biological activity of adenovirus is their transgene expression capacity. Unlike live virus vaccines where achievement of infection is the goal, the aim of an adenovector is to induce expression of a heterologous gene product (Altaras et al., 2005). To evaluate whether the transgene expression capability of virus was preserved after lyophilization, the luciferase and endostatin expression was detected in A549 cells and CNE-1 cells separately, with freshly purified adenovirus stock as control.

Fig. 5A shows that after freeze drying, the luciferase expression level of adenovirus containing luciferase gene in formulations S1 and I1 was recovered to 58 and 76%, respectively. By comparison, the transgene expression capability in formulations C and M was mostly lost. A similar phenomenon was also observed in the endostatin assay. From Fig. 5B, it can be found that in comparison with freshly purified adenovirus, formulations S1 and I1 recovered 45 and 55% of the endostatin protein expression level respectively indicating that the endostatin protein was efficiently secreted into the supernatant of transduced CNE-1 cells. Thus, the transgene expression capacity of E10A in terms of luciferase and endostatin protein expression was well preserved when viruses were freeze dried in formulation I1. These results highly correspond with the infectivity assay suggesting that defects in capsid assembly or disruptions in protein integrity which affect delivery of the adenovirus genome to the nucleus, thereby abolishing infectivity, should affect transgene expression proportionately (Altaras et al., 2005).

3.5. Stability of E10A formulations during storage

E10A samples, lyophilized in formulations S1 and I1 were stored at 37 °C and 4 °C in sealed vials. The stability of the lyophilized powder was evaluated by monitoring the infectivity during storage for 28 days at 37 °C and 180 days at 4 °C, using viruses dispersed in solution and stored under the same conditions as a reference. As we can see from Fig. 6A, E10A solution lost more than 99.9% infectivity after 3 weeks of storage at 37 °C. In contrast, E10A lyophilized in formulations S1 and I1 maintained 29% and 66% of the original activity over a period of 4 weeks, respectively. In long-term stability study, we observed that the E10A solution stored at 4 °C only retained less than 3% of initial infectivity. On the contrary, formulations S1 and I1 allowed storage at 4 °C for up to 180 days where 83% and 89% of the starting activity was retained, respectively, after an initial loss of less than 50% due to lyophilization processing. These results indicate that application of lyophilization technology in the presence of excipients, specifically the mannitol and inulin combinations considerably improved the stability of E10A during storage at 4 °C as well as 37 °C.

4. Conclusion

In this work, a grid of formulation and process parameters important for stabilizing the E10A during lyophilization were determined and a stable E10A lyophilized powder was successfully prepared. The freezing and primary drying temperatures for the lyophilization process were determined via the evaluation of critical formulation temperatures including T_{eu} and T_c. The lyophilization of an optimized formulation (formulation I1) yielded a stable E10A lyophilized powder with preserved infectivity, no adenoviruses aggregates, maintained transgene expression capacity and adequate storage stability. In addition, it was found the amorphous matrix formed by mannitol combinations with sucrose or inulin was indispensable for the stabilization of E10A during lyophilization. Hence, the lyophilized E10A formulations with eliminated glycerol toxicity and improved stability will probably enhance the applicability of E10A for tumor gene therapy.

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