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Stabilization of gene delivery systems by freeze-drying

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Abstract

Freeze-drying of three different forms of gene delivery systems was performed using a controlled two-step drying process and 10% sucrose as lyoprotectant. Complexes of pCMVL plasmid with transferrin-conjugated polyethylenimine, adenovirus-enhanced transferrinfection consisting of pCMVL/transferrin-polylysine complexes linked to inactivated adenovirus particles, and a recombinant, E1-defective adenovirus expressing a luciferase reporter gene were tested. Three weeks after freeze-drying the reagents were rehydrated with water and tested for transfection activity. Luciferase gene expression levels were retained at high levels in all three systems, in contrast to reagents stored in solution. The use of the lyoprotectant was essential. In the absence of sucrose the transfection activities dropped by a factor of 100–1000. The data suggest freeze-drying as a useful method for stabilization and storage of standardized batches of transfection agents. © 1997 Elsevier Science B.V.

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1. Introduction

Gene therapy is strongly influenced by any progress in the development of viral and non-viral vectors that are safe, deliver the therapeutic gene efficiently into the patient's target cells, and can be generated under standardized conditions in compliance with pharmaceutical and regulatory requirements. Non-viral vectors (Cotten and

Abbreviations: pCMVL = Plasmid DNA encoding the *Photinus pyralis* luciferase as reporter gene; CMV = cytomegalovirus; RPMI = a commerical available cell culture medium from Life Technologies: RPMI stands for Rossman-Park-Memorial-Institute; PBS = phosphate buffered saline.

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Wagner, 1993; Ledley, 1995), based on naked plasmid DNA or DNA complexes with cationic lipids or polycationic polymers, are attractive because of their defined chemistry, low cost, and high flexibility.

Polyionic gene transfer complexes consisting of two or more components are usually freshly prepared shortly before transfection of the cells. This is thought to be required due to the physical instability of complexes formed under kinetically rather than thermodynamically controlled conditions. These complexes are susceptible to aggregation or growth of particle size which can impair gene delivery function. The drawbacks of day-to-day preparation of complexes include the danger of batch to batch variations, and due to time constraints, the inability to perform extensive quality control before the actual transfection. Freeze-drying would be an obvious approach for stabilization and storage of large standardized batches. We report here, methods for freeze-drying three different forms of gene delivery reagents assembled into transfection-competent form. These include DNA complexed with polyethylenimine-transferrin (Kircheis et al., 1997); DNA complexed with polylysine linked to an adenovirus carrier (Wagner et al., 1992) and a recombinant adenovirus. Unlike the aqueous forms of these materials, the freeze-dried forms are suitable for long term storage. Transfection-ready material is obtained by the simple addition of water. The ability to prepare such samples is an important step in converting molecular biology tools into clinic-friendly drugs.

2. Materials and methods

2.1. Transfection agents

The recombinant adenovirus pAdLuc was generated using a two plasmid system (Bett et al., 1994), grown in 293 cells (Graham et al., 1977) and purified by double banding in CsCl gradients. The virus bears the CMV-driven luciferase unit from pCMVL (Plank et al., 1992) inserted in the E1 region. For freeze-drying, 200 μl of the virus (0.36×10^{12} particles/ml) was diluted with 1.8 ml HBS (HEPES-buffered saline: 150 mM NaCl, 20 mM HEPES, pH 7.3). Half of the solution was diluted with 1 ml of HBS, the other half was diluted with 1 ml of 20% sucrose.

The plasmid pCMVL (Plank et al., 1992) coding for the *Photinus pyralis* luciferase gene, was purified by column chromatography according to the manufacturers instructions (Nucleobond, Macherey-Nagel) and treated to remove lipopolysaccharide (LPS) as described (Cotten et al., 1994a). The lipopolysaccharide content of plasmids was measured by the Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD) and was less than 0.02 endotoxin units/μg DNA. Transferrin polyethylenimine conjugate Tf-PEI, containing transferrin (Biotest, Germany) and polyethylenimine (Fluka, Switzerland, average molecular weight of 800 kDa) at a molar ratio of 2:1, were synthesized as described in (Kircheis et al., 1997). Transferrin-polylysine conjugates (molar ratio of 1:1) and streptavidin-polylysine conjugates (molar ratio of 1:1) were prepared as described (Wagner et al., 1991, 1992) using polylysine (Sigma, hydrobromide, average molecular weight of 50 kDa), streptavidin (Pierce) and transferrin (Biotest).

2.2. Cell culture reagents

Media and fetal calf serum (FCS) were purchased from Gibco-BRL (Gaithersburg, MD). The human erythromyeloid leukemia cell line K562 (ATCC CCL-243) was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in RPMI 1640 medium/

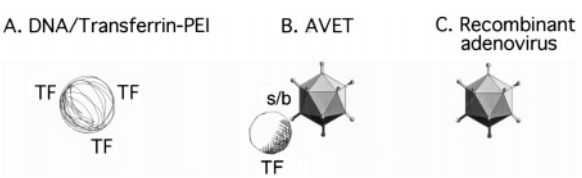

Fig. 1. Transfection systems tested in the lyophilization protocol. *A*: plasmid DNA (pCMVL) condensed with transferrin-conjugated polyethylenimine; *B*: plasmid DNA condensed with transferrin-polylysine and streptavidin-polylysine linked to biotinylated/psoralen-inactivated dl1014 adenovirus; *C*: recombinant adenovirus pAdLuc.

10% FCS. Primary human skin fibroblasts were used between passage 5 and 15 and grown in DMEM, 10% FCS (DMEM, 2 mM glutamine, 100 IU penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal calf serum).

2.3. Preparation of dna/transferrin-polyethylenimine complexes

Transfection complexes were prepared similarly as described by Kircheis et al. (1997). Plasmid DNA pCMVL (200 μg) and transferrin-conjugated polyethylenimine (180 μg) were separately diluted in each 5 ml HBS. The solutions were then mixed and incubated at room temperature for 30 min. Half (5 ml) of the complex solution was diluted with 5 ml of HBS, the other half was diluted with 5 ml of 20% sucrose yielding a final concentration of 10 μg DNA per ml in either buffer or 10% sucrose solution.

2.4. Preparation of adenovirus/DNA/transferrin-polylysine complexes

Transfection complexes were prepared as described by Wagner et al. (1992). Biotinylated, methoxypsoralen inactivated adenovirus dl1014 (Bridge and Ketner, 1989; Cotten et al., 1994b; 600 μl stock solution, approximately 2×10^{11} particles) in 2 ml HBS was mixed with 6 μg of streptavidin-polylysine in 2 ml HBS. After a 20 min incubation, 120 μg pCMVL in 3 ml HBS were added and incubated for 20 min at room temperature. The mixture was combined with 100 μg transferrin-polylysine conjugate in 3 ml HBS followed by a further incubation for 20 min. Half (5 ml) of the complex solution was diluted with 5 ml of HBS, the other half was diluted with 5 ml of 20% sucrose.

Table 1
Water content of DNA complexes

Complexes		Water content	
Solution	Time (h)	(%)	S.D.
HBS	4	0.21	0.02
HBS	24	0.28	0.12
HBS/Sucrose	4	4.38	0.05
HBS/Sucrose	24	2.84	0.76

Complexes dissolved in HBS or in $0.5 \times$ HBS/10% sucrose were lyophilized at -40°C followed by a 4 or 24 h drying period at room temperature. Water content of samples was determined as described in Materials and methods. S.D., standard deviation.

2.5. Freeze-drying of complexes

Transfection samples were freeze-dried in 1 ml aliquots. The following freeze-drying protocol was applied. A Leybold GT4 pilot-plant production freeze-dryer was used. During freeze-drying in the secondary drying phase the vials could be individually stoppered by a custom-built stoppering device. The 1 ml vials partially closed with rubber freeze-drying stoppers were frozen at a pre-cooled shelf at -40°C for at least 30 min. In the first step of the freeze-drying process (primary step) the plate temperature was maintained at -40°C and the chamber pressure at 10–13 Pa for approximately 60 h. In the second step (the secondary drying phase) the temperature was raised with 10°C per hour until 20°C finally reaching a

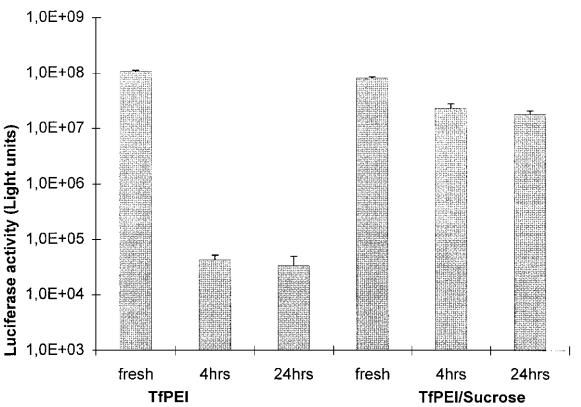

Fig. 2. Transfection of K562 cells by lyophilized DNA/transferrin-polyethylenimine complexes. DNA complexes were prepared and used for transfection as described in Material and methods. Lyophilized complexes (prepared 3 weeks before the transfection, freeze-dried from HBS or in $0.5 \times$ HBS/10% sucrose solutions, with a 4 or 24 h secondary drying period at 20°C) were compared with the corresponding freshly prepared DNA complexes. All samples represent 10 μg pCMVL DNA per well. Transfection efficiency is expressed as total light units per well and shown as average $\pm$ standard deviation of triplicates.

pressure of circa 1 Pa. During the secondary phase the vials were closed to obtain samples with differing amounts of residual water. The condensor temperature ranged between −50°C and −55°C during the entire freeze-drying process. Lyophilized samples were stored in the cold (at 4°C and −20°C) or on dry ice during shipment between laboratories.

2.6. Determination of residual water contents

The residual water content was determined with the Karl-Fisher method using a Mitsubishi moisture meter model CA-05 (Tokyo, Japan). To minimize exposure of the hygroscopic cakes to the environment, the rubber caps remained on the vials throughout the sampling procedure. Cakes were dissolved in 1 ml of Hydranal Coulomat A (Riedel de Haen, Seelze, Germany) which was injected through the rubber caps. The water content of 100 μl aliquots of the solvent was measured in duplicates and expressed as % (w/w).

2.7. Transfection of cells

K562 cells were plated in 24 well plates (Nunc, Roskilde, Denmark) at a density of 5×10^5 cells per well in 1.5 ml RPMI culture medium containing 10% fetal calf serum. Lyophilized DNA complexes were dissolved in 1 ml sterile water per vial and added to the cells within 30 min. For comparison, freshly prepared DNA complexes were used. After 4 h incubation, the transfection medium was replaced with fresh culture medium. Cells were harvested 24 h after transfection and luciferase activity was determined (Cotten et al., 1993). In short, cells were washed in PBS, resuspended in 100 μl Tris (0.25 M, pH 7.5), and lysed by three freeze-thaw cycles. The cell lysate was centrifuged for 5 min at 10 000 g to pellet debris. Luciferase light units were recorded (using a Lumat LB9501/16 instrument from Berthold, Bad Wildbad, Germany) from an aliquot of the supernatant (5–10 μl) with 10 s integration after automatic injection of the luciferin solution. Luciferase background (300–400 light units) was subtracted from each value and the transfection efficiency was expressed as total light units per well.

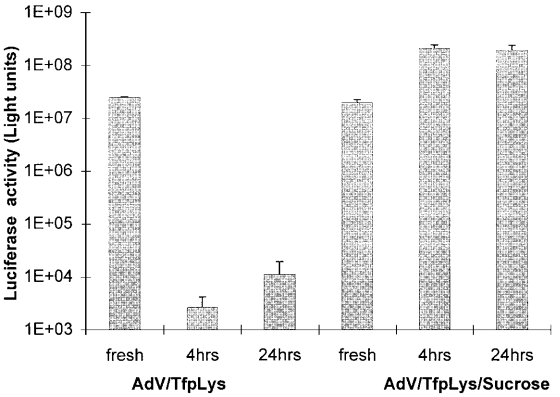

Fig. 3. Transfection by adenovirus-enhanced transferrin-polylysine DNA complexes. DNA complexes were prepared and used for transfection of K562 cells as described in Materials and methods. Lyophilized complexes (3 weeks old) were compared with freshly prepared DNA complexes. All samples represent 6 μg pCMVL DNA per well. Transfection efficiency is expressed as total light units per well and shown as average ± standard deviation of triplicates.

3. Results and discussion

Three different efficient transfection systems have been tested in the lyophilization protocol (Fig. 1). The first is a non-viral complexes of pCMVL plasmid DNA (coding for the *Photinus pyralis* luciferase gene) with transferrin-conjugated polyethylenimine (Kircheis et al., 1997). This is a newly developed transfection reagent which combines the DNA delivery capacity of the synthetic polycation polyethylenimine (Boussif et al., 1995) with the receptor targeting properties of transferrin (Wagner et al., 1991) system. The second system is the chimeric adenovirus-enhanced transferrinfection system (AVET) consisting of DNA/transferrin-polylysine complexes linked to inactivated adenovirus particles (Wagner et al., 1992). The third system is a recombinant, E1-defective adenovirus expressing a luciferase reporter gene.

A pilot-plant production freeze-dryer was used which allows freeze-drying under well-defined and controllable conditions (Van Winden and Crommelin, 1997). Lyophilization was performed in two steps, with a primary drying phase at −40°C and a secondary phase at +20°C. The transfection agents were freeze-dried from the standard

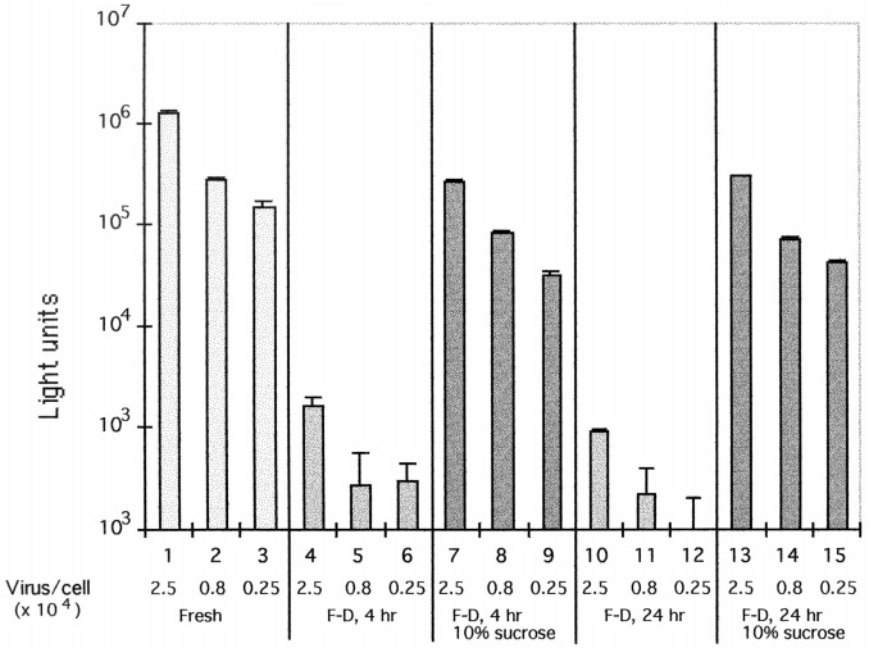

Fig. 4. Transduction by recombinant adenovirus. Purified ADLuc1 was tested as either a 'fresh' virus (after storage at -70°C in 40% glycerol) or after freeze-drying in HBS in the presence or absence of 10% sucrose, and secondary drying at 20°C for 4 or 24 h (see Materials and methods). The lyophilized virus samples were redissolved in the apprpriate volume of water and tested for gene delivery activity into primary human fibroblasts. Cells (20 000 cells/well, 24 well dish in 0.5 ml of DMEM/2% horse serum) were exposed to virus at the indicated virus to cell ratio (25 000, 8000 and 2500 virus particles/cell) for 2 h at 37°C. The medium was replaced with DMEM/10% FCS and 24 h later luciferase activity was measured. The average of three transduction samples (with a standard deviation) is indicated.

salt solution (HBS) or after addition of sucrose (10% final concentration). The water content of DNA complexes lyophilized at −40°C followed by a 4 or 24 h secondary drying period at 20°C are shown in Table 1. Samples lyophilized from HBS contained only traces of water at both 4 and 24 h, whereas sucrose-containing samples had significant water contents of about 3–4%. The complexes were rehydrated after 3 weeks and compared in their transfection activity with freshly prepared transfection agents (Figs. 2 and 3). Luciferase gene expression of freeze-dried pCMVL/transferrin-polyethlenimine complexes was retained, but only when sucrose was included in the formulation; in the absence of sucrose the expression dropped by at least 3 log units (Fig. 2). The activity of transfection solutions stored for 3 weeks at 4°C or room temperature was strongly reduced (by 200–1000-fold) and formation of large aggregates was observed as measured by light scattering (data not shown). The addition of sucrose did not alter the activity of freshly prepared transfection complexes. Cryoprotectants like sucrose have the ability to form a protective sugar-glass during freezing of the solution. Pure water crystallizes and the solutes are concentrated in the remaining network that after removal of the ice in the primary drying-phase, is shown as a freeze-drying cake. During secondary drying, the residual water in the cake is removed which increases the glass-transition temperature and the stability of the cake.

Freeze-drying gave very similar results with the adenovirus-enhanced AVET system (Fig. 3). Interestingly, the gene expressions of lyophilized complexes were found to be slightly higher than freshly prepared complexes, the reasons for this are not clear. No difference between the two

secondary drying times (4 and 24 h) were observed.

The results of subjecting recombinant adenovirus pAdLuc to freeze-drying are shown in Fig. 4, approx. 25% of activity was retained with the sucrose formulation, whereas less than 0.25% of the activity was recovered in the absence of sucrose.

4. Conclusion

In conclusion, a sucrose-containing formulation was found to be suitable for freeze-drying of three different transfection systems. A similar formulation has been developed for lyophilization of DNA nanoparticles (Cherng et al., 1996) with poly(2-dimethylamino)ethyl methacrylate (J.Y.C., H.T., W.H. and Daan Crommelin, in preparation). The current work demonstrates that gene transfer activity of lyophilized complexes was retained after storage for 3 weeks. Further studies will be required to determine the long term stability and physico-chemical characteristics of the freeze-dried DNA complexes.

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