Freeze-Drying of Poly((2-dimethylamino)ethyl Methacrylate)-Based Gene Delivery Systems

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INTRODUCTION

Recently, we reported on the use of poly(2-dimethylamino)ethyl methacrylate (PDMAEMA) as a non-viral transfectant (1,2). This polymer is able to bind electrostatically to plasmids yielding polymer-plasmid complexes, which are able to be taken up by cells. It was found that the size of the formed complexes was a dominant factor for the transfection efficiency. The highest transfection efficiency was observed at a polymer/plasmid ratio of 3 (w/w) and weight average molecular weight of the polymer above 250 kD. Under these conditions particles with a size between 0.15 and 0.25 μm and a slightly positive zeta-potential were formed. However, the complexes have a limited stability in an aqueous solution due to possible chemical and physical degradation processes. E.g. we have recently found that complexes aged at 40°C for 2 months showed a decrease in transfection efficiency of about 50% (Cherng et al, to be published). Therefore, methods have to be developed to stabilise these structures to fulfil the shelf-life requirements defined for pharmaceutical products. Freeze-drying is a frequently used process to stabilise labile therapeutic agents, among which proteins (3–5) and drug loaded liposomes (6). Until now, no systematic studies have been published dealing with freeze-drying of non-viral gene delivery systems. The aim of this study is to evaluate the possibilities to preserve the transfection potential of PDMAEMA/plasmid complexes by freeze-drying and to gain insight into the factors affecting this process.

MATERIALS AND METHODS

Materials

pCMV-lacZ plasmid contains a bacterial lacZ gene preceded by a Nuclear Location Signal under control of the CMV promoter (1,7). 2-(Dimethylamino)ethyl methacrylate (DMAEMA) was obtained from Fluka. RPMI-1640 medium and DMEM (Dulbecco's modified Eagles medium) were obtained form Gibco, Breda, The Netherlands. Foetal Calf Serum (FCS) was purchased from Integron, Zaandam, The Netherlands. Cells were cultured in complete DMEM medium, which was prepared by supplementing plain DMEM with FCS (final concentration 5%), Hepes (final concentration 25 mM, pH 7.4), penicillin (final concentration 100 IU/ml), streptomycin (final concentration 100 μg/ml) and amphotericin B (final concentration 0.25 μg/ml). X-Gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) was from Gibco, Breda, The Netherlands. Sucrose and maltose-monohydrate were obtained from Merck, Germany. D(+)-Trehalose-dihydrate was from Sigma, Sigma Chemical Co., England. All other chemicals and reagents were of analytical grade.

Synthesis and Characterization of PDMAEMA

PDMAEMA was prepared by a radical polymerisation of 2-(dimethylamino)ethyl methacrylate essentially as described previously (1). The number average molecular weight (M_n) and weight average molecular weight (M_w) relative to dextran as determined by gel permeation chromatography were $47 * 10^3$ and $280 * 10^3$ g/mol, respectively.

Preparation of PDMAEMA-plasmid Particles

PDMAEMA was dissolved in Hepes (20 mM, pH 7.4) to a concentration of 300 μg/ml. The plasmid stock solution was diluted to a concentration of 400 μg/ml in the same buffer. Next, 100 μl plasmid solution was mixed with 500 μl aqueous solution of the lyoprotectant (sucrose, trehalose or maltose). Subsequently, 400 μl polymer solution was added and gently mixed for 5 seconds (Vortex Genie 2). Thus, the solutions finally contained Hepes 10 mM (2.4 mg/ml), PDMAEMA 0.12 mg/ml, plasmid 0.04 mg/ml, and varying concentration of lyoprotectant ranging from 12.5 to 200 mg/ml. After 30 minutes incubation at ambient temperature, the formed complexes were either frozen or evaluated for their transfection efficiency.

Particle Size Measurements

The z-average particle size and polydispersity index (p.d.) of the PDMAEMA-plasmid particles were determined by dynamic light scattering (DLS) at 25°C with a Malvern 4700 system using 25 mW He-Ne laser (NEC Corp., Tokyo, Japan) and the automeasure version 3.2 software (Malvern Ltd, Malvern, UK). For the data analysis, the viscosity (0.8905 mPa.s) and refractive index (1.333) were used for Hepes at 25°C. When lyoprotectants were present, viscosity and refractive index were corrected.

Freeze-Drying and Freeze-Thawing

Aliquots of PDMAEMA-plasmid dispersions of 1 ml were filled in 10 ml glass vials and frozen for 60 minutes by placing them on the shelf (pre-cooled by circulating silicone oil of −40°C) of a Leybold GT4 pilot-production freeze-dryer. The set pressure was 16 Pa which corresponds to the vapor pressure of ice at about −38°C. During the primary drying process, the temperature was kept below the collapse temperature of sucrose (−32°C) (8). After 40 hours, the shelf temperature was raised to +20°C with a rate of 10 °C/hour and kept at this temperature

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(under a pressure of 1Pa) for 2 hours. Finally, the vials were closed with a closing device under vacuum. The freeze-dried polymer/plasmid particles stored at 4°C were rehydrated and evaluated for size and transfection efficiency within 10 days.

For the freeze-thawing, samples were frozen according to the same procedure, but thawed in a water bath of 37°C before analysis.

Moisture Analysis

Residual moisture contents of the freeze-dried cakes were determined by the Karl Fisher method using a Mitsubishi moisturemeter (model CA-05, Tokyo, Japan).

MDSC Analysis

The glass transition temperatures (T_g) of the freeze-dried cakes were determined by modulated differential scanning calorimetry (MDSC) (9,10). In order to minimise attraction of water by the hygroscopic freeze-dried cakes, sampling was performed in a dry nitrogen gas environment. Samples of the freeze-dried cakes were punched out and transferred into aluminium pans, which were closed tightly. T_g-values were determined by taking the half-height between the baseline above and below the temperature range of the glass transition. All scans were recorded with a DSC 2920 (TA instrument, Inc., New Castle, DE, USA), equipped with a liquid nitrogen cooling accessory.

Rehydration of the Freeze-Dried Cakes

After freeze-drying, the cakes were dissolved in 1 ml water and the size of the polymer/plasmid complexes was determined by DLS. Subsequently, 7 ml RPMI-1640 medium was added resulting in a final concentration of 15 and 5 μg/ml for the polymer and plasmid, respectively.

Cell Culture and Transfection

For the gene transfer studies, COS-7 cells (cells of SV-40-transformed African green monkey kidney) were used essentially as described previously (1). The cells were seeded in a flat-bottom 96-well plate (1×10^4 cells per well (0.38 cm^2)) 24 hours before transfection in complete DMEM. Thereafter, the PDMAEMA-plasmid complexes (volume 200 μl) were added to the cells and incubated for 1 hour at 37°C and 5% CO_2. After removal of the transfection complexes, the cells were cultured for an additional 2 days in complete DMEM and then evaluated for transfection efficiency.

Expression of the pCMV-lacZ gene was established by incubation of fixed cells (0.25% glutaraldehyde; 5 minutes; 4°C) with X-gal solution (0.8 mg/ml in phosphate buffer pH 7.4) for 24 hours at 37°C. Using a light microscope, transfected cells were made visible as blue spots and were quantified by counting the number of blue spots in each well. Transfection values were normalised to the number of transfected cells found after incubation of the cells with freshly prepared polymer/plasmid complexes in Hepes (relative transfection efficiency).

RESULTS AND DISCUSSION

We selected a simple buffer solution (20 mM Hepes, pH 7.4) to prepare the transfection complexes. DLS experiments showed that the particle size of these complexes was not significantly different from sizes of particles prepared in RPMI (0.15–0.25 μm) (1,2). Since it has been reported that sucrose might affect the in vitro transfection (11), it was our aim to keep the concentrations of sugars as low as possible during the incubation of the cells with the transfection complexes. In a control experiment, it was shown that 2.5% (w/v) sucrose in RPMI did not influence the transfection efficiency of freshly prepared polymer/plasmid complexes. At higher sucrose concentrations a slight increase in number of transfected cells was observed. Therefore, to exclude the effect of sucrose on transfection, we reconstituted the cakes in a total volume of 8 ml, yielding a sugar concentration not exceeding 2.5%, and a polymer and plasmid concentration of 15 and 5 μg/ml, respectively, which corresponds with conditions where the transfection showed an optimum (1,2).

In the first series of experiments polymer/plasmid complexes were prepared with a varying concentration of sucrose (0–5%). After freeze-thawing, the size and transfection efficiency of the complexes were evaluated. Fig. 1 shows that in the absence of sucrose, large particles were found which showed a reduced transfection efficiency. On the other hand, in the presence of sucrose both the particle size and the transfection potential of the complexes was preserved.

All freeze-dried samples (except the formulation without lyoprotectant) had good cakes which readily dissolved in water. Fig. 2 shows that after rehydration of the freeze-dried samples prepared at a low sucrose concentration (1.25%), large particles were present. Obviously, this amount of sucrose is not enough to prevent aggregation of the polymer/plasmid complexes. In agreement with previous studies (1,2), these large complexes possessed a relatively low transfection efficiency. Combining Fig. 1 and 2, it can be concluded that at this low sucrose concentration the damage to the complexes occurs during the drying process. Fig. 2 also shows that small particles were detected after rehydration of the freeze-dried cakes with 1 ml water, which were obtained after freeze-drying of aqueous polymer/plasmid solutions containing a concentration of sucrose ≥ 2.5% . No substantial change in particle size was observed after the addition of 7 ml RPMI. Moreover, the trans-

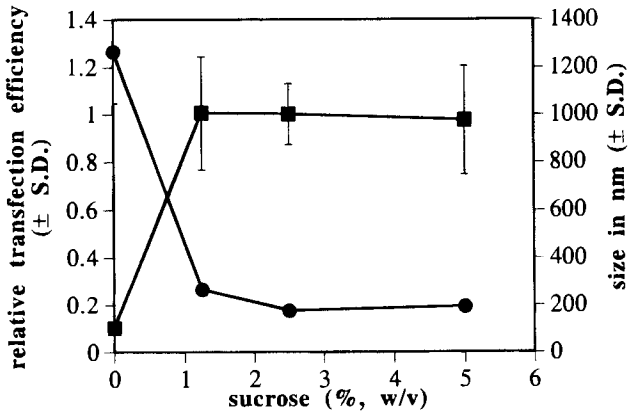

Fig. 1. Size (●) and relative transfection efficiency (■) of polymer/plasmid complexes after freeze-thawing. The % sucrose refers to the concentration present in the aqueous polymer/plasmid solution used for the preparation. The results are expressed as mean values ±S.D. of 3–5 experiments.

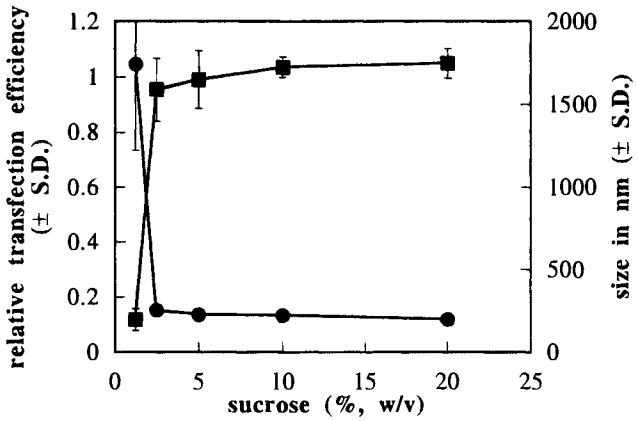

Fig. 2. Size (●) and relative transfection efficiency (■) of polymer/plasmid complexes after freeze-drying and rehydration. The % sucrose refers to the concentration present in the aqueous polymer/plasmid solution used for the preparation of the cakes. The results are expressed as mean values ±S.D. of 3–5 experiments.

fection efficiency of these particles was not significantly different from freshly prepared particles.

After freeze-drying, the resulting cakes were evaluated for their residual water content and glass transition temperature. Fig. 3 shows that the residual water content of the cakes increased with sucrose concentration used for the preparation of the cakes. This increase in residual water content is associated with a decrease in the glass transition temperature. The freeze-dried formulation with a low concentration of sucrose (1.25%) contained a relatively high amount of Hepes (16% w/w). However, cakes with and without Hepes showed the same Tg. This means that Hepes in the cakes is possibly present in the crystalline state (T_m = 234°C). The glass transition temperature of the different sucrose matrices followed the well known Fox-equation:

$$1/Tg = (W_1/T_{g1}) + (W_2/T_{g2})$$

Where T_g is the glass transition temperature of the sucrose matrix; W_1 and W_2 are the weight fraction of component 1 (sucrose) and 2 (water); T_{g1} is the glass transition temperature of component 1 (sucrose, 335 K) and T_{g2} is the glass transition temperature of component 2 (water, 138 K). This result suggests that the residual water is uniformly distributed over the sugar matrix. Although the transfection efficiency of the polymer/plasmid complexes was not dependent on the residual water content of the freeze-dried cakes, this might be an important factor when evaluating the long-term stability of freeze-dried transfection complexes.

Knowing that sucrose is an effective lyoprotectant for polymer/plasmid complexes, we compared its performance with two other frequently used lyoprotectants (trehalose and maltose). In Fig. 4 is shown that for the investigated sugars, freeze-drying and freeze-thawing gives the same transfection efficiency. DLS experiments demonstrated (data not shown) that the size of the transfection complexes only slightly increased from around 0.18 μm (directly after preparation) until approx. 0.22 μm after freeze-thawing and freeze-drying. This increase in particle size was associated with a slight increase in the polydispersity index, suggesting that some limited aggregation had occurred. Interestingly, the type of lyoprotectant had no effect on either the size or the transfection efficiency of the complexes after freeze-drying and freeze-thawing. Aggregation of the complexes in the sugar matrices might be prevented due to the glassy character of the matrices and/or specific interactions (e.g. H-bonding) between the sugars and the polymer/plasmid complexes. However, other factors like physical separation of the complexes in the sugar matrices also might contribute to the observed stability.

In conclusion, this paper shows that freeze-drying is an excellent method to preserve the size and transfection potential of polymer/plasmid complexes. The type of lyoprotectant (sugar) used is of minor importance. However, the concentration of the sugars is an important factor affecting both the size and transfection capability of the complexes after freeze-drying and freeze-thawing. If there is a damage of polymer/plasmid complexes during freeze-drying, it results from the drying process but not due to the freezing step. Presently, we are evaluating the long-term stability of freeze-dried polymer/plasmid complexes.

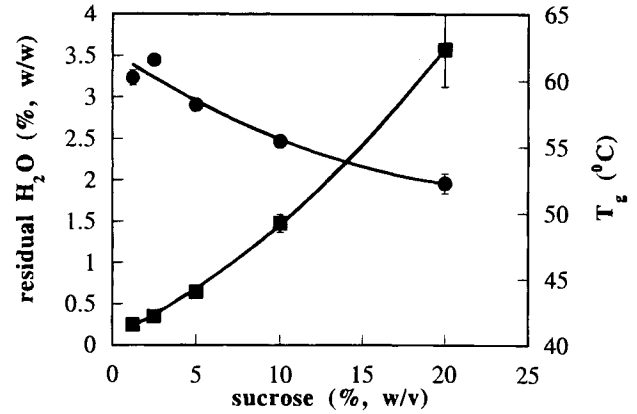

Fig. 3. Residual water content (■) and T_g (●) of sucrose cakes also containing polymer/plasmid complexes. The % sucrose refers to the concentration present in the aqueous polymer/plasmid solution used for the preparation of the cakes. The results are expressed as mean values of 2–3 experiments.

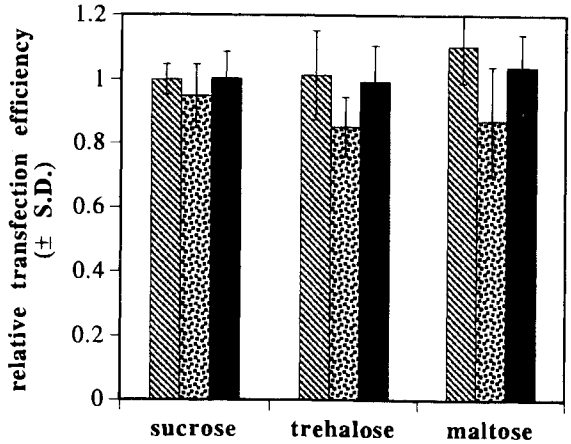

Fig. 4. Relative transfection efficiency of polymer/plasmid complexes prepared with different sugars (10 %, w/v): -freshly prepared (▨), -after freeze-drying and rehydration (▦) -after freeze-thawing (■). The results are expressed as mean values ±S.D. of 3–5 experiments.

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