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Strategies for maintaining the particle size of peptide DNA condensates following freeze-drying

Kai Y. Kwok[a], Roger C. Adami[a], Kelly C. Hester[a], Youmie Park[b], Steve Thomas[a], Kevin G. Rice[a,b,*]

[a] *Divisions of Pharmaceutics, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065, USA*
[b] *Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109-1065, USA*

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Abstract

The particle size of peptide DNA condensates were studied after freeze-drying and rehydration as a function of sugar excipient, concentration, pH, DNA concentration, and peptide condensing agent. In the absence of an excipient, freeze-dried 50 μg/ml AlkCWK$_{18}$ (iodoacetic acid alkylated Cys-Typ-Lys$_{18}$) DNA condensates formed large fibrous flocculates on rehydration. Of the sugars tested as lyoprotectants, sucrose proved most effective at preserving particle size during rehydration. The addition of 5 wt/vol% sucrose preserved a mean particle diameter of less than 50 nm during rehydration of AlkCWK$_{18}$ DNA condensates prepared at DNA concentrations up to 200 μg/ml; however, higher DNA concentrations led to the formation of insoluble fibrous flocculates. Substitution of polyethylene glycol (PEG)-CWK$_{18}$ as a DNA condensing peptide eliminated the need for sucrose, resulting in peptide DNA condensates that retained particle size when rehydrated in water or normal saline at concentrations up to 5 mg/ml. The results suggest that sucrose functions primarily as a bulking agent during freeze-drying that only preserves the particle size of AlkCWK$_{18}$ DNA condensates up to a maximum concentration of 200 μg/ml. Alternatively, the steric layer created on the surface of PEG-CWK$_{18}$ DNA condensates provides far more efficient lyoprotection, preserving their particle size at a concentration of 5 mg/ml without a bulking agent.

Keywords: Peptide DNA condensates; Freeze-drying; Lyoprotectant; Polyethylene glycol; Particle size; Solubility; Aggregation prevention

1. Introduction

The development of controlled release gene delivery formulations is an attractive approach to sustain gene expression at or near an implantation site. Manufacturing these delivery systems using well-established solvent evaporation methods ap-

* Corresponding author. Tel.: +1-734-7631032; fax: +1-734-7632022.
E-mail address: krice@umich.edu (K.G. Rice).

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plicable to small hydrophobic drugs presents a serious problem when formulating DNA due to its poor solubility in organic solvents. One recently demonstrated solution involved lyophilizing plasmid DNA with granular polylactide glycolic acid (PLGA) followed by gas foaming in carbon dioxide to form a PLGA sponge (Shea et al., 1999).

To further improve this controlled release formulation, peptide DNA condensates could be substituted for naked plasmid DNA. This is likely to enhance the gene delivery performance of PLGA sponges by reducing DNA metabolism (Adami et al., 1998) and increasing DNA uptake into cells (Wadhwa et al., 1997). However, incorporating peptide DNA condensates into PLGA sponges is problematic due to the tendency of condensed DNA to aggregate on freeze-drying (Ma et al., 1998), creating large particles that are poorly internalized into cells (Anchordoquy et al., 1997; Cherng et al., 1997; Wadhwa et al., 1997; Bettinger et al., 1999). Hence we investigated the influence of lyoprotectants on the colloidal particle size of peptide DNA condensates following freeze-drying and rehydration.

Several earlier studies have investigated the relationship between particle size and transfection efficiency after lyophilization of nonviral gene delivery systems (Anchordoquy et al., 1997; Cherng et al., 1997; Leong et al., 1998; De Jaeghere et al., 1999). In the absence of excipients, lyophilized and rehydrated lipid/DNA complexes lost approximately 70% of their ability to transfect cells in culture (Anchordoquy et al., 1997). The addition of sucrose or trehalose preserved the size of lipid/DNA complexes allowing recovery of the transfection efficiency, leading these authors to propose that sugars may replace water as hydrogen bond partners during the freeze-drying of lipid/DNA complexes, as they have been shown to do so for liposomes and proteins (Crowe et al., 1985; Allison et al., 1999). Similar results from Cherng et al. demonstrated that sucrose also protected polymer-based DNA complexes from aggregation during freeze-drying, which rehydrated to produce equivalent gene expression as freshly prepared samples (Cherng et al., 1997). More recently, Anchordoquy et al. showed that sucrose protected lipid/DNA complexes during rapid freeze-thawing resulting in retention of particle size and transfection efficiency (Anchordoquy et al., 1998).

Two earlier reports addressed the use of sugars as lyoprotectants to maintain the particle size of freeze-dried and rehydrated polyethylenimine (PEI) (Talsma et al., 1997) or peptide DNA condensates (Ma et al., 1998). Talsma et al. found that 10 wt/vol% sucrose was necessary to recover full transfection potency of freeze-dried PEI-transferrin DNA condensates. Ma et al. also reported that 1 wt/vol% sucrose and mannitol preserved the rehydrated particle size of dilute solutions of fibroblast growth factor (FGF-II) polylysine$_{80}$ DNA condensates and allowed rehydration to higher concentrations of DNA condensates (Ma et al., 1998).

In the present study, we have investigated the influence of excipient, concentration, pH, DNA concentration, and peptide condensing agent in an attempt to preserve peptide DNA condensate particle size following rehydration. Consistent with the results of earlier studies, sucrose was found to be the best lyoprotectant to maintain the particle size of dilute (50–200 μg/ml) peptide DNA condensates but failed to lyoprotect DNA condensates above this concentration. Here we report that a PEG-peptide conjugate remarkably improved the rehydration properties of freeze-dried peptide DNA condensates, thus preserving the particle size following rehydration of DNA condensates at concentrations as high as 5 mg/ml. This advancement should allow the convenient formulation of PEG-peptide DNA condensates with PLGA following dry mixing.

2. Materials and methods

2.1. Materials

Plasmid NT-βGal (7.5 kb) was produced in *E. coli* and purified using a Qiagen Ultrapure-100 kit (Santa Clarita, CA). AlkCWK$_{18}$ and PEG-CWK$_{18}$ were prepared as reported previously (Wadhwa et al., 1997; Kwok et al., 1999). Glucose, mannitol, galactose, dextran (500 kDa), su-

crose, sodium chloride and Hepes were purchased from Sigma, (St. Louis, MO). PEG-VS (5 kDa) was purchased from Fluka (Ronkonkoma, NY).

2.2. Formulation and freeze-drying of peptide DNA condensates

The concentration of $AlkCWK_{18}$ and PEG-CWK_{18} were determined by UV absorbance (Trp, $\varepsilon_{280\,nm} = 5600$ M/cm). Peptide DNA condensates were prepared in 5 mM Hepes pH 5 at a peptide/DNA stoichiometry of 0.3 nmol of peptide/μg of DNA. The condensates were formed by adding 450 μl of 100 μg/ml NT-βGal drop wise to 450 μl of 30 nmol/ml $AlkCWK_{18}$ or PEG-CWK_{18} while vortexing. Each sample was prepared in triplicate, and after a 30 min equilibration, 20–100 μl of 50 wt/vol% solutions of either glucose, mannitol, galactose, dextran or sucrose were added followed by normalization to 1 ml to give a final sugar concentration of 1–5 wt/vol% and DNA concentration of 50 μg/ml.

The mean particle size of 50 μg/ml $AlkCWK_{18}$ DNA condensates was determined by quasielastic light scattering (QELS) on a Brookhaven Zeta-Plus. Samples (1 ml) were frozen rapidly in dry ice ethanol in a 1.5 ml microcentrifuge tube and then lyophilized on a Labconco™ freeze dryer for 24 h operated at a constant vacuum of 55×10^{-6} mBar while maintained at room temperature (22°C). The maximal vacuum was achieved within 2 min after applying the sample. At the highest sucrose concentration of 5 wt/vol%, samples remained frozen throughout freeze-drying but dried to form a glassy solid after 24 h. Samples possessing 1 wt/vol% sucrose dried to a crystalline powder whereas PEG-peptide DNA condensates dried to form a light fluffy powder. Freeze-dried samples prepared in triplicate were reconstituted in 1 ml of deionized water, allowed to equilibrate for 30 min and then analyzed by QELS as described above. Large peptide DNA condensates were measured by light microscopy with magnification of $\times 100$. The particle size of PEG-peptide DNA condensates were also measured by transmission electron microscopy (TEM) according to a published procedure (Wadhwa et al., 1997).

The relationship between pH and rehydrated peptide DNA condensate particle size was examined in 5 mM Hepes at a pH range of 3–7. The particle size of 50 μg/ml DNA condensates in 5 wt/vol% sucrose was determined by QELS before and after freeze-drying as described above.

The rehydrated particle size of $AlkCWK_{18}$ or PEG-CWK_{18} DNA condensates were also examined at a concentration of 0.05–5 mg/ml in 5 mM Hepes pH 5 with ($AlkCWK_{18}$ DNA) or without (PEG-CWK_{18} DNA) the addition of 5 wt/vol% sucrose. DNA condensates were freeze-dried and reconstituted to the original concentration. QELS particle size analysis before and after freeze-drying required dilution of an aliquot of the sample to a concentration of 50 μg/ml. Freeze-dried 5 mg/ml PEG-CWK_{18} DNA condensates were also reconstituted in 0.9 wt/vol% sodium chloride and analyzed for particle size.

3. Results and discussion

The objective of this study was to investigate parameters to maintain peptide DNA condensate particle size when rehydrated following freeze-drying. This is especially important when designing controlled release drug delivery systems that lypophilize DNA with PLGA prior to gas foaming (Shea et al., 1999) since the formation of large flocculates during freeze-drying or rehydration of condensed DNA in the PLGA sponge would significantly reduce the gene transfer efficiency.

Our initial observations indicated that rehydration of freeze-dried $AlkCWK_{18}$ DNA condensates resulted in large fibrous flocculates observed by light microscopy to be approximately 100 μm in diameter (Fig. 1A). This apparently occurs either during dehydration or rehydration but not during rapid freezing since the particle size of freeze-thawed $AlkCWK_{18}$ DNA condensates, with or without added sucrose, were only slightly larger than controls. Likewise, increasing the rehydration volume did not decrease the degree of flocculation suggesting that either the flocculates form very rapidly during rehydration or when concentrated during dehydration.

Other studies have noted that sugar excipients serve as lyoprotectants to preserve the particle size of lipid/DNA and polymer/DNA complexes (Cherng et al., 1997; Anchordoquy et al., 1998; De Jaeghere et al., 1999), which may result from both their ability to bulk DNA complexes and through direct hydrogen bonding to the surface of DNA complexes. To establish if sugar excipients could also suppress the formation of flocculates formed on freeze-drying peptide DNA condensates, we analyzed the ability of different sugar excipients added at 5 wt/vol% to preserve the particle size of 50 μg/ml $AlkCWK_{18}$ DNA condensates on rehydration. The results presented in Fig. 1 demonstrate that sucrose, dextran and galactose were equally effective in preserving the rehydrated peptide DNA condensate particle size, whereas glucose and mannitol afforded slightly larger DNA condensates (Fig. 1). As discussed in more detail below, these subtle differences in the lyoprotection afforded to peptide DNA condensates by different sugars are most likely related to their bulking properties rather than to differences in the degree of hydrogen bonding to the surface of peptide DNA condensates.

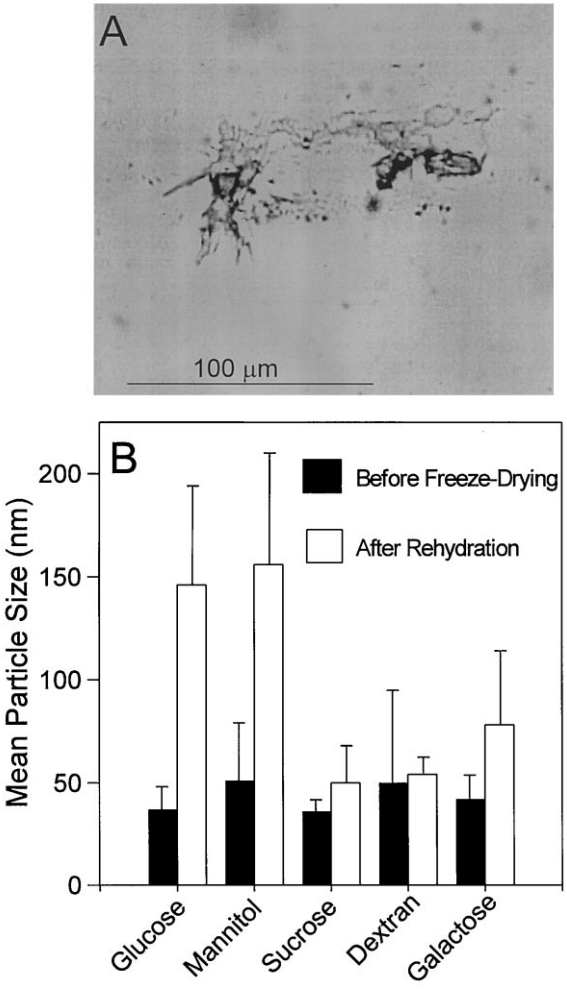

Fig. 1. The effect of different sugar excipients on preserving $AlkCWK_{18}$ DNA condensate particle size following freeze-drying and rehydration to 50 μg/m1. The light micrograph in panel A illustrates 100 μm flocculates formed following freeze-drying and rehydration of 50 μg/ml $AlkCWK_{18}$ DNA condensates without lyoprotectant. Panel B illustrates the QELS particle size for peptide DNA condensates prepared with sugar excipients at a concentration of 5 wt/vol% in 5 mM Hepes pH 5. Each sample was analyzed in triplicate and produced a mean particle diameter of less than 50 nm before freeze-drying (closed bar). After freeze-drying and rehydration (open bar) a significantly ($P < 0.05$) larger particle size was determined when using glucose or mannitol as a lyoprotectant relative to sucrose.

It is important to note that throughout these studies multimodel QELS particle size analysis typically yielded two populations of particles composed of a dominant (95 vol%) population with diameters ranging from 30 to 45 nm and a minor population of 130–180 nm representing approximately 5 vol%. The data in Figs. 1–5 represent the mean diameter based on averaging the size and intensity of these two populations.

Peptide DNA condensates composed of a single 7.2 kb plasmid (4.44×10^6 g/mol) bound by approximately 757 peptides (assuming a 1:1 NH_4:PO_4 ratio and each peptide is 2672 g/mol, total = 2.02×10^6 g/mol), have a calculated molecular weight of 6.46×10^6 g/mol. Assuming collapse into spherical particles of the same density (5.9×10^{-22} g/nm^3) as a protein (Sugio et al., 1999), they could occupy a minimal diameter of 32 nm. Under the same assumptions, if two peptide condensed plasmids are contained in a single particle they would occupy a minimal particle size of 40 nm, whereas four plasmids would represent a particle of 52 nm. Based on this calculation, the majority of peptide DNA condensates are assumed to be multiplexes containing from one to four plasmids that make up the dominant 30–45 nm

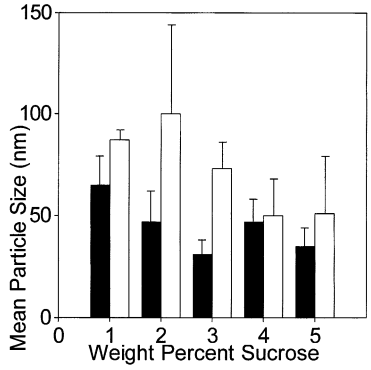

Fig. 2. The effect of sucrose concentration on preserving $AlkCWK_{18}$ DNA condensate particle size after freeze-drying and reconstitution to 50 μg/ml. The peptide DNA condensates were prepared with varying sucrose concentration from 1 to 5 wt/vol% in 5 mM Hepes pH 5. Each sample was analyzed in triplicate and the mean particle diameter ± standard deviation before (closed bar) and after freeze-drying and rehydration (open bar) is reported. There was no statistical significance in the rehydrated particle size when using 1–5 wt/vol% sucrose as a lyoprotectant.

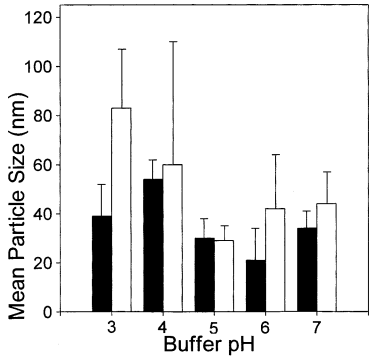

Fig. 3. The effect of pH on preserving $AlkCWK_{18}$ DNA condensate particle size after freeze-drying. Peptide DNA condensates were prepared in 5 mM Hepes buffer with pH ranging from 3 to 7 at a sucrose concentration of 5 wt/vol%. Each sample was analyzed in triplicate and the mean particle diameter ± standard deviation before (closed bar) and after freeze-drying and rehydration (open bar) is reported. The rehydrated particle size at pH 3 was significantly ($P < 0.05$) larger than that determined at pH 5 whereas the rehydrated particle size at pH 4, 6, or 7 was not significantly larger than 5.

population of particles whereas a smaller percentage appear to be fused multiplexes with diameters of 130–180 nm. An increase in mean diameter on rehydration was typically accompanied by an increase in both the mean diameter and intensity (vol%) of the fused multiplex population whereas the formation of flocculates results from the aggregation of many fused multiplexes.

The results presented in Fig. 2 demonstrated the rehydrated particle size of $AlkCWK_{18}$ DNA condensates was not influenced by decreasing the sucrose concentration from 5 to 1 wt/vol%. However, sucrose concentrations less than 1 wt/vol% resulted in the formation of flocculates following rehydration (not shown) suggesting that 1 wt/vol% is the minimum sucrose concentration to effectively bulk 50 μg/ml peptide DNA condensates during freeze-drying.

In addition to the concentration of the sugar excipient, the pH of the buffer may, influence the particle size of rehydrated peptide DNA condensates by affecting the affinity of peptide binding to DNA or the surface properties (Kabanov and Kabanov, 1998). However, the data in Fig. 3 demonstrate that the particle size of rehydrated $AlkCWK_{18}$ DNA condensates were not strongly influenced by pH. Likewise, the zeta potential only decreased from + 35 mV at pH 7 to + 30 mV at pH 3 establishing that the surface charge only changed slightly by lowering the pH.

Analysis of the rehydrated particle size as a function of $AlkCWK_{18}$ DNA condensate concentration revealed an upper limit of 200 μg/ml below which the particle size was maintained (Fig. 4) and above which gave rise to large fibrous flocculates that were measured by light microscopy to be approximately 100 μm in diameter (Fig. 4A). Thereby, the proposed bulking effect of 5 wt/vol% sucrose that lyoprotects peptide DNA condensates at dilute concentrations (50 μg/ml) appears insufficient at even moderately higher DNA concentrations. These results are also consistent with a primary mechanism involving sucrose as a bulking agent and not as a hydrogen bond partner as noted for proteins and liposomes (Crowe et al., 1985; Allison et al., 1999) since this later mechanism would not be expected to be sensitive to small differences in peptide DNA concentration.

Since bulking agents such as sucrose are apparently limited in their ability to lyoprotect DNA condensates, an alternate strategy is to directly

modify the colloid surface of peptide DNA condensates to block inter-particle interactions that lead to the formation of flocculates during dehydration. We have previously reported PEG-CWK_{18} as a unique DNA condensing peptide that decreases the zeta potential to +10 mV and forms DNA condensates of less than 100 nm up to DNA concentrations of 2 mg/ml (Kwok et al., 1999). To determine if PEG-CWK_{18} could also stabilize the particle size of freeze-dried DNA condensates, the relationship between DNA condensate concentration and particle size following freeze-drying and rehydration was examined. The results presented in Fig. 5 establish that PEG-CWK_{18} preserved the particle size of freeze-dried and rehydrated 5 mg/ml DNA condensates without the addition of sucrose as a bulking agent. TEM analysis of rehydrated 5 mg/ml PEG-CWK_{18} DNA condensates also confirmed the presence of only small particles that appeared to be less than 100 nm in diameter (Fig. 5A). We hypothesize that the hydrated flexible PEG chains provide steric stabilization by producing a dense layer over the particle surface that prevents peptide DNA condensate aggregation during dehydration. In support of this hypothesis, the covalent attachment of PEG to the surface of DNA condensates was found to be essential, since the addition of 10-fold excess (300 μg) PEG-VS (5 kDa) to preformed AlkCWK_{18} DNA condensates failed to alter their zeta potential or afford lyoprotection.

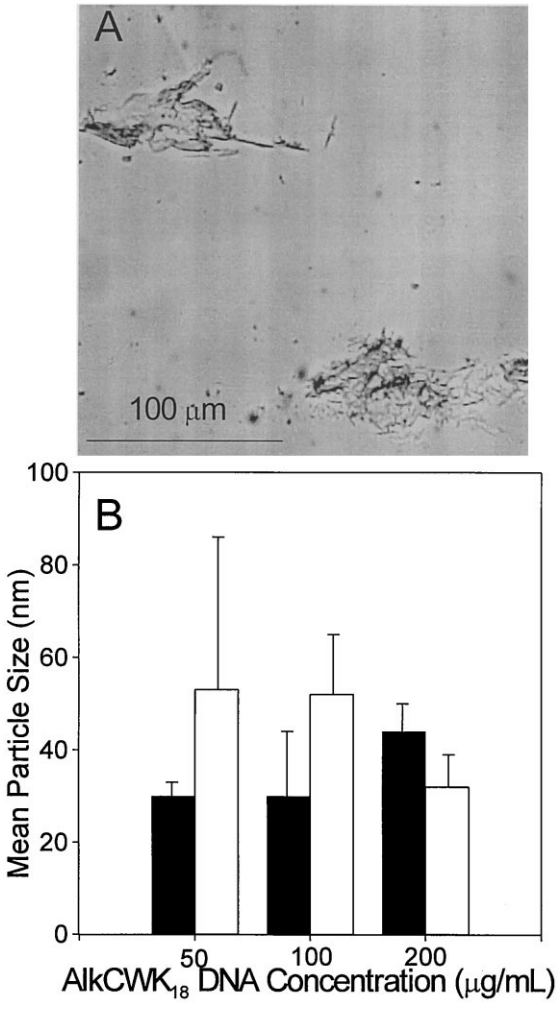

Fig. 4. The effect of DNA concentration on particle size after freeze-drying and rehydration. The light micrograph in panel A illustrates 100 μm flocculates formed following freeze-drying and rehydration of 400 μg/ml AlkCWK_{18} DNA condensates prepared in 5 mM Hepes pH 5 containing 5 wt/vol% sucrose. The results in panel B compare the QELS particle size of AlkCWK_{18} DNA condensates prepared at concentrations varying from 50 to 200 μg/ml in the same lyoprotectant. Each sample was analyzed in triplicate and the mean particle diameter ± standard deviation before (closed bar) and after freeze-drying and rehydration (open bar) is reported. At low DNA concentrations (< 200 μg/ml) there was no statistical significance in the rehydrated particle size whereas the particle size grew very large at higher concentrations as indicted by the light micrograph in panel A.

The finding that the particle size of freeze-dried 5 mg/ml PEG-CWK_{18} DNA condensates were even preserved when rehydrated in normal saline (Fig. 5) established the value of this approach in formulating isotonic dosage forms. These results also suggest PEG-CWK_{18} DNA condensates will likely rehydrate to form small particles following implantation of a PLGA sponge containing encapsulated condensed DNA.

We conclude that concentrations below 200 μg/ml of AlkCWK_{18} DNA condensates can be freeze-dried and rehydrated while preserving particle size provided that a lyoprotectant such as 5 wt/vol% sucrose is included to serve primarily as a bulking agent. In contrast, the lyoprotection afforded using PEG-CWK_{18} as a condensing peptide preserved the particle size of freeze-dried 5 mg/ml DNA condensates when rehydrated in

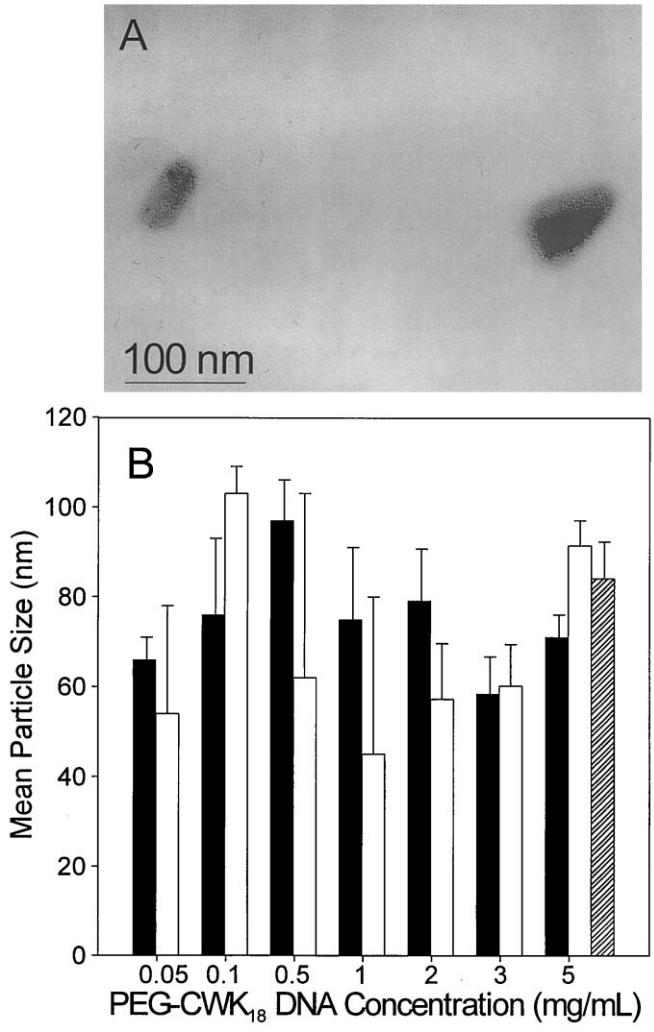

Fig. 5. Particle size of PEG-CWK_{18} DNA condensates following freeze-drying. The DNA concentration of PEG-CWK_{18} DNA condensates was varied from 0.05 to 5 mg/ml in 5 mM Hepes pH 5. Each sample was analyzed in triplicate and the mean particle diameter $\pm$ standard deviation before (closed bar) and after freeze-drying and rehydration (open bar) is reported in panel B. PEG-CWK_{18} DNA condensates prepared at 5 mg/ml were also freeze dried and reconstituted in normal saline (striped bar) prior to measuring particle size. There was no statistical significance in the particle size of rehydrated PEG-CWK_{18} DNA condensates across the concentration range of 0.05–5 mg/ml. Panel A illustrates TEM of 5 mg/ml PEG-CWK_{18} DNA condensates that were freeze-dried and rehydrated in water resulting in small (< 100 nm) particles.

water or normal saline without a bulking agent. These data support a proposed mechanism involving modification of the DNA condensate surface by creating a steric layer that blocks inter-particle interactions during dehydration and rehydration, resulting in a far more efficient lyoprotection relative to the application of bulking agents. The results of this study will be valuable in developing advanced delivery systems for peptide DNA condensates in which dry mixing of freeze-dried DNA colloids is desired as a formulation step.

Acknowledgements

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References

Adami, R.C., Collard, W.T., Gupta, S.A., Kwok, K.Y., Bonadio, J., Rice, K.G., 1998. Stability of peptide-condensed plasmid DNA formulations. J. Pharm. Sci. 87, 678–683.

Anchordoquy, T., Carpenter, J., Kroll, D., 1997. Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration. Arch. Biochem. Biophys. 348, 199–206.

Anchordoquy, T., Girouard, L., Carpenter, J., Kroll, D., 1998. Stability of lipid/DNA during agitation and freeze-thawing. J. Pharm. Sci. 87, 1046–1051.

Allison, S.D., Chang, B., Randolph, T.W., Carpenter, J.F., 1999. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Arch. Biochem. Biophys. 365, 289–298.

Bettinger, T., Remy, J.-S., Erbacker, P., 1999. Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. Bioconj. Chem. 10, 558–561.

Cherng, J.-Y., Wetering, P.V.D., Talsma, H., Crommelin, D.J.A., Hennink, W.E., 1997. Freeze drying of poly((2-dimethylamino)ethyl methacrylate)-based gene delivery systems. Pharm. Res. 14, 1838–1841.

Crowe, L.M., Crowe, J.H., Chapman, D., 1985. Interaction of carbohydrates with dry dipalmitoylphophatidylcholine. Arch. Biochem. Biophys. 2-36, 289–296.

De Jaeghere, F., Allemann, E., Leroux, J.-C., et al., 1999. Formulation and lyoprotection of poly(lactic acid-co-ethylene oxide) nanoparticles: influence on physical stability and in vitro cell uptake. Pharm. Res. 16, 859–866.

Kabanov, A.V., Kabanov, V.A., 1998. Interpolyelectrolyte and block ionomer complexes for gene delivery: physicochemical aspects. Adv. Drug Delivery Rev. 30, 49–60.

Kwok, K.Y., McKenzie, D.L., Evers, D.L., Rice, K.G., 1999. Formulation of highly soluble poly(ethylene glycol)-peptide DNA condensates. J. Pharm. Sci. 88, 996–1003.

Leong, K.W., Mao, E.-Q., Truong-Le, V.L., Roy, K., Walsh, S.M., August, J.T., 1998. DNA-polycation nanospheres as non-viral gene delivery vehicles. J. Cont. Rel. 53, 183–193.

Ma, C., Chen, A.X., Ying, W., D'Andrea, M.J., 1998. Stable liquid and lyophilized formulations of polycation: DNA complexes. Pharm. Sci. (Suppl.) 1, 288.

Shea, L.D., Smiley, L., Bonadio, L., Mooney, D.J., 1999. DNA delivery from polymer matrices for tissue engineering. Nat. Biotechnol. 17, 551–554.

Sugio, S., Kashima, A., Mochizuki, S., Noda, M., Kobayashi, K., 1999. Crystal structure of human serum albumin at 2.5 Å resolution. Protein Engineering 12, 439–446

Talsma, H., Cherng, L.-Y., Lehrmann, H., et al., 1997. Stabilization of gene delivery systems by freeze-drying. Int. J. Pharm. 157, 233–238.

Wadhwa, M.S., Collard, W.T., Adami, R.C., McKenzie, D.L., Rice, K.G., 1997. Peptide mediated gene delivery: influence of peptide structure on gene expression. Bioconj. Chem. 8, 81–88.