JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 301–306

Received October 10, 2002
Accepted November 29, 2002
DOI 10.1211/002235702702
ISSN 0022-3573

The effect of protective agents on the stability of plasmid DNA by the process of spray-drying

Jung-hua Steven Kuo

Abstract

The effect of several protective agents was assessed on the stability of spray-dried plasmid DNA. The spray-drying process had adverse effects on the tertiary structure of plasmid DNA with the protective agents of sucrose, glycine and agarose. With the protection of these noncondensing agents, a band corresponding to the linear form of plasmid DNA was observed in the gel electrophoresis between the supercoiled circular (SC) form and the open circular (OC) form. On the contrary, spray-dried plasmid DNA maintained some degree of structural integrity under the protection of condensing agents. For the protection by neutral condensing polymers, such as polyethylene glycol 1000 and 4000, no linear form between the SC form and the OC form of plasmid DNA was revealed in the gel electrophoresis. Also, excess cationic condensing polymer, polyethyleneimine, had the ability to provide the plasmid DNA with protection from degradation as indicated by the preservation in SC and OC forms of plasmid DNA on the agarose gel electrophoresis. Moreover, DNA topology was unchanged after six-month storage at 4 °C by the protection of these neutral and cationic condensing agents. Accordingly, DNA condensation induced by condensing agents may provide a way to minimize damage to plasmid DNA by the process of spray drying.

Introduction

One of the fastest growing research areas involves plasmid DNA for gene therapy (Miller 1992). To obtain optimal biological effectiveness, plasmid DNA has to retain its supercoiled circular (SC) and open circular (OC) forms (Kimoto & Taketo 1996). In addition, all aspects of regulatory review must comply with the most rigorous standards to ensure the plasmid DNA products remain in a defined and characterized isoform. Therefore, the degradation of the SC and OC forms is considered strictly undesirable. The degradation of plasmid DNA occurs in any aqueous solution near neutral pH by the two-step process of depurination and β-elimination, leading to cleavage of the phosphodiester backbone (Suzuki et al 1994). The introduction of structural changes will eventually convert the SC form of plasmid DNA to the OC and linear forms (Middaugh et al 1998). The stability of plasmid DNA is dependent on many factors, including temperature and shear stress (Lindahl & Karlstrom 1973; Levy et al 1999).

The spray-drying process has recently been used to develop aerosol delivery for biotechnology-based pharmaceuticals (Maa et al 1998). The unique features of spray drying lie in its ability to involve both particle formation and drying in a single-step (Master 1991). As a result, a fast spray-drying process may represent a subject of increasing interest in the area of the treatment of pulmonary diseases such as cystic fibrosis and lung cancer. Nonetheless, spray-dried biotechnology-based products may encounter a loss of biological activity due to certain structural alterations as a result of the processing environment. Besides thermal degradation, the instability of biopharmaceuticals during the process of spray drying has been attributed to shearing stresses in the nozzle and adsorption at the liquid/air interface of the spray solution on atomization (Niven et al 1994; Maa & Hsu 1997). In general, protective agents such as disaccharides and polyols included in the solution are essential to protect the biological integrity of biopharmaceuticals during spray drying (Labrude et al 1989; Broadhead et al 1994). The stabilizing mechanism is primarily due to interactions, such

Department of Pharmacy, Chia Nan University of Pharmacy and Science, 60 Erh-Jen Rd., Sec. 1, Jen-Te, Tainan 717, Taiwan, ROC

Jung-hua Steven Kuo

Correspondence: J.-h. S. Kuo, Department of Pharmacy, Chia Nan University of Pharmacy and Science, 60 Erh-Jen Rd., Sec. 1, Jen-Te, Tainan 717, Taiwan, ROC. E-mail: ph0510@yahoo.com

Acknowledgements: The author would like to thank the National Science Council of the Republic of China (NSC 91-2216-E-041-001) for supporting this research.

as hydrogen bonding, between biopharmaceuticals and protective agents (Carpenter & Crowe 1989). The study of the stability profile of spray-dried plasmid DNA formulations is limited. Spray-dried DNA powders containing trehalose have been prepared, but no details of DNA topology analysis were reported (Freeman & Niven 1996).

The major purpose of this study was to evaluate the effectiveness of protective agents to obtain stable plasmid DNA to avoid degradation by the spray-drying process. The various noncondensing protective agents used were sucrose, glycine, and agarose. The neutral condensing protective agents studied were polyethylene glycol 1000 and 4000 whereas the cationic condensing protective agent studied was polyethyleneimine. Agarose gel electrophoresis was used to analyse the effectiveness of the protective agents on the stability of plasmid DNA by the process of spray drying.

Materials and Methods

Materials

The plasmid, pCMV-Luc, contains the luciferase reporter gene under control of the CMV promoter. pCMV-Luc was amplified in *Escherichia coli* (strain DH5α) and purified by column chromatography (QIAGEN-Mega kit, Netherlands). The purity of pCMV-Luc was established by UV spectroscopy (E260 nm/E280 nm ratio ranging from 1.87 to 1.89 was used). Agarose (0.7%) gel electrophoresis analysis using restriction enzymes showed that pCMV-Luc was mainly in the supercoiled form and one band corresponding to a size of 7.8 kb was visible. Reagent grade of sucrose, polyaspartic acid and glycine were obtained from Sigma Chemical Company (St Louis, MO) and used without further purification. Polyethylene glycol (PEG) 1000 and 4000 were purchased from Fluka Chemie GmbH (Switzerland). Polyethyleneimine (PEI, MW = 800 kDa) was obtained from Sigma as 50% (w/v) solution. The PEI solutions were adjusted to desired aqueous concentrations and neutralized with HCl (pH = 7.0). Molecular biology grade agarose was supplied by Vegonil (Moreno Valley, CA).

Spray-drying

The spray-drying process was performed using a Büchi 190 mini spray dryer (Switzerland). Atomizing air (6 bar) and feed solution passed separately to the two-fluid 0.5-mm pneumatic nozzle in co-current flow systems. The feed solution (pump setting = 2) was prepared by dispersing 1 mg pCMV-Luc and an appropriate amount of protective agent in 25 mL deionized water, and this was sprayed at various preset inlet/outlet temperatures. The aspirator setting was kept at the scale of 12 and the airflow rate was 700 NL h^{-1} during the process. The dried particles were separated from the hot air stream in a cyclone and collected in a receiving vessel. The noncondensing agents used in the spray drying were sucrose (20 and 30%), glycine (5%) + sucrose (20%), and agarose (2%). The condensing agents used were PEG 1000 (20%), PEG 4000 (20%), and PEI-DNA (3/1 and 5/1 w/w) in a 20% sucrose solution.

Agarose gel electrophoresis

After spray drying, the dried powders (1 μg pDNA/well; 10 μL + 2 μL Type IV loading buffer/well) in TAE buffer (pH = 8.0) were loaded onto a 0.7% agarose gel containing ethidium bromide and underwent electrophoresis at 100 V for 1 h. After electrophoresis, DNA was examined by UV-irradiation. For PEI cationic polymers, polyaspartic acid solution (25 mg mL^{-1}) was used to dissociate the cationic polymer–DNA complexes at ambient temperature. The resulting compound was incubated for 24 h.

Results and Discussion

The agarose gel electrophoresis of spray-dried plasmid DNA in 20% sucrose after processing at various outlet temperatures is shown in Figure 1. As compared with control plasmid DNA (Figure 1, lane 1), a sharp decrease of the SC band was observed under all processing temperatures. For all these spray-dried samples, a band corresponding to the linear form of plasmid DNA was observed in the gel between the SC form and the OC form. The results indicated that the integrity of plasmid DNA was changed by the spray-drying process, even when the outlet processing temperature was decreased to as low as 56 °C (Figure 1, lane 2). The typical temperature difference between hot drying air and droplet surface during spray drying was approximately 15 °C (Master

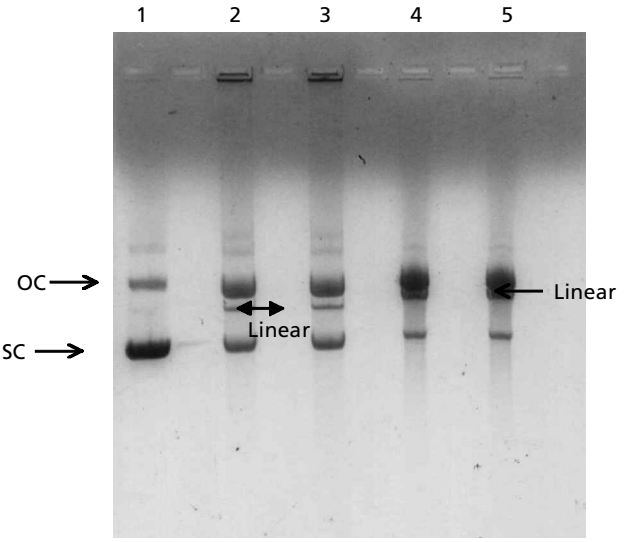

Figure 1 Agarose gel electrophoresis of spray-dried plasmid DNA at various outlet temperatures. Lane 1: control plasmid DNA (without spray drying); lanes 2–5: spray-dried plasmid DNA (1 mg pCMV-Luc in 20% sucrose solution) at outlet temperatures of 56, 69, 94, and 122 °C (± 2 °C), respectively. The band position for supercoiled circular (SC), open circular (OC) and linear DNA is indicated with arrows.

1991). At this temperature (around 40 °C), without spray drying, plasmid DNA in aqueous solution remained in the SC and OC forms (data not shown). Accordingly, the shear stress and air–liquid interfaces of plasmid solution may result in the structural change of plasmid DNA (Maa et al 1998). Also, previous observations indicated that large molecules of DNA were easily broken by shear stress (Reese & Zimm 1990). The observation is consistent with previous studies that the induced shear stress can lower the activation energy required for plasmid DNA degradation (Adam & Zimm 1977).

To circumvent the stability issue raised by temperature and shear stress, the effects of sucrose alone and of sucrose combined with glycine on the stability of plasmid DNA were studied (Figure 2). Similarly, the 30% sucrose (lane 2) had no significant improvement on the structural integrity of plasmid DNA after spray drying. In addition to sucrose, glycine has been reported also as a protective agent for some protein-based products (Walsh 1998). Glycine was incorporated into the 20% sucrose solution containing 1 mg plasmid DNA at concentrations of 5% (lane 3). Nevertheless, a linear band was still evident under these mild spray-drying conditions, which suggested that the combination of sucrose and glycine was not sufficient to protect the plasmid DNA from structural change.

To minimize damage to DNA by shear stress attempts were made to introduce interactions between DNA and protective agents in the solution (Sambrook & Russel 2001). For 2% agarose (lane 4), a band corresponding to the linear form of plasmid DNA was observed in the gel between the SC form and the OC form. Apparently, agarose was ineffective to protect the plasmid DNA from structural change by the spray-drying process although DNA was successfully protected in agarose blocks in most molecular biology experiments. Figure 3 shows the effect of several other protective agents on the stability of plasmid DNA after spray drying. For PEG 1000 and 4000 (lanes 3 and 4, respectively), no linear form of plasmid DNA was observed in the gel between the SC form and the OC form. As compared with control plasmid DNA, a slight decrease of the SC band was observed under the process of spray drying. This evidence demonstrated that spray-dried plasmid DNA mostly retained structural integrity under the protection of PEG 1000 and 4000. This protective effect of two PEGs may be attributed to the DNA condensation by neutral crowding polymers such as PEG through an excluded volume mechanism (Vasilevskaya et al 1995; Bloomfield 1996). That is, the dramatic decrease in plasmid DNA volume provoked by PEG may contribute to the minimized DNA damage by shear stress during spray drying. The protective effect of cationic polymers on the stability of plasmid DNA by the process of spray drying was evaluated in Figure 3. Similarly, previous reports have shown that the cationic polymers may interact with the negatively charged larger piece of DNA and thus the two types of molecules

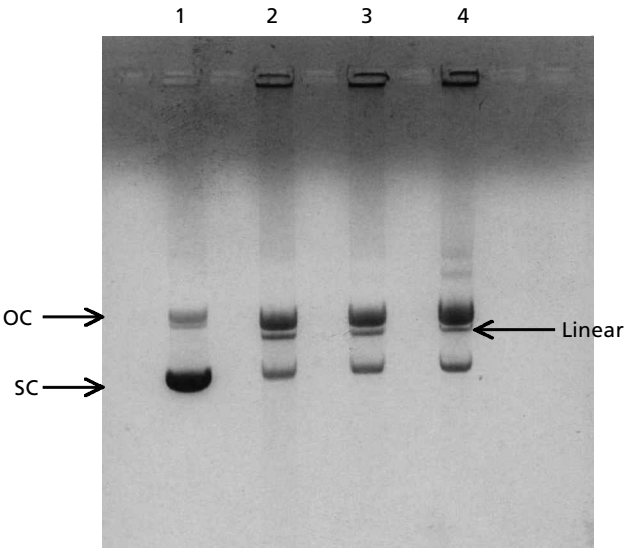

Figure 2 Agarose gel electrophoresis of spray-dried plasmid DNA under the protection of noncondensing agents. Lane 1: control plasmid DNA; lanes 2 and 3: spray-dried plasmid DNA (1 mg pCMV-Luc) in 30% sucrose and 5% glycine/20% sucrose solutions, respectively (outlet temperature = 56 ± 2 °C); lane 4: spray-dried plasmid DNA in 2% agarose solution. The band position for supercoiled circular (SC), open circular (OC) and linear DNA is indicated with arrows.

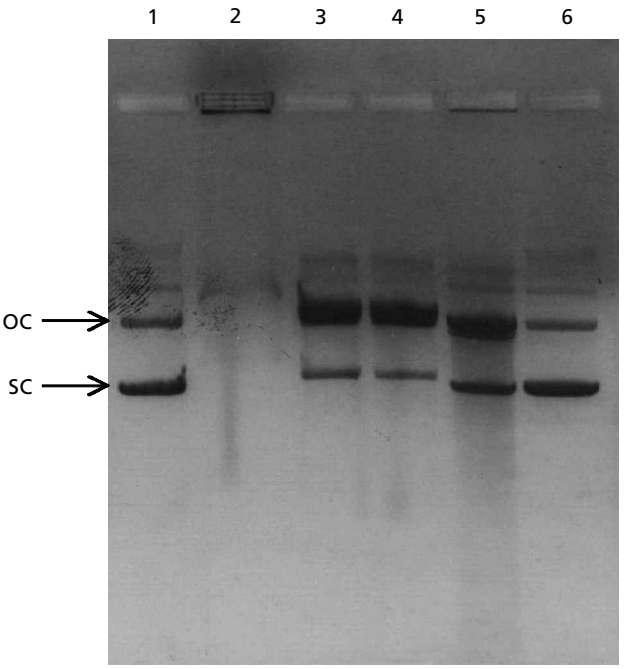

Figure 3 Agarose gel electrophoresis of spray-dried plasmid DNA under the protection of condensing agents. Lane 1: control plasmid DNA; lanes 3 and 4: spray-dried plasmid DNA (1 mg pCMV-Luc) in 20% (w/v) PEG 1000 and 20% (w/v) PEG 4000 solutions, respectively; lane 2: spray-dried plasmid DNA in 20% sucrose solution (PEI/DNA (w/w) 5/1) without polyaspartic acid dissociation; lanes 5 and 6: spray-dried plasmid DNA in 20% sucrose solution (PEI/DNA (w/w) 3/1 and 5/1, respectively) with polyaspartic acid dissociation. The outlet temperature = 56 ± 2 °C. The band position for supercoiled circular (SC) and open circular (OC) DNA is indicated with arrows.

may condense to compact complexes in the aqueous solutions (Boussif et al 1995; Gebhart & Kabanov 2001). Furthermore, electrostatic forces may lead to a higher denaturation temperature of plasmid DNA and transition temperature of polymer for the cationic polymer–plasmid DNA complexes (Kuo et al 2002). The above two mechanisms suggest the addition of cationic polymers may provide the protection of plasmid DNA to withstand the thermal and shear stress during spray drying. Accordingly, the complexation of plasmid DNA with PEI was probed in aqueous solution with cationic polymer/DNA weight ratios of 5/1 and 3/1. The choice of PEI/DNA ratios (3 and 5 w/w) was based on the optimal transfection at the above ratios on several cell lines. For typical PEI–DNA complexes, no band was revealed in the agarose gel electrophoresis after spray drying in aqueous solution (lane 2). To distinguish DNA from the resulting complexes in the agarose gel, the resulting complex solution was treated with excess polyaspartic acid to dissociate cationic polymers (Trubetskoy et al 1999). Indeed, for excess cationic polymer/DNA ratios (5/1 and 3/1), no linear form was revealed in the agarose gel electrophoresis between the SC form and the OC form after the process of spray drying, as shown in Figure 3 (lanes 5 and 6). As reported previously, the transfection efficiency in several cell lines was much higher for SC/OC plasmid DNA than for linearized plasmid DNA (Xie & Tsong 1993; Adami et al 1998). However, the protective effect of excess DNA/cationic polymer ratio might not be sufficient to condense the plasmid DNA from structural degradation, as demonstrated by previous studies that complexation of excess DNA with the cationic polymers resulted in a decreased thermal stability of SC DNA (Lobo et al 2002). The above results demonstrated that the condensation induced by condensing agents such as PEG and cationic polymers may provide the ability to protect plasmid DNA from structural degradation by the process of spray drying. In an effort to understand the protective mechanism by polymers, our results demonstrated that the interactions between polymer and plasmid DNA was critical to withstand the shear stress generated by the process of spray drying. Treatment of plasmid DNA with neutral crowding polymers such as PEG induced DNA condensation and this kind of interaction with DNA was sufficient to resist the DNA degradation. In addition to condensation, cationic polymers also generate electrostatic forces with the negatively charged larger piece of DNA and were effective to provide the DNA stability under spray drying.

To examine the long-term stability of plasmid DNA and to evaluate the possible reversibility of conformational change process, the spray-dried plasmid DNA under the protection of noncondensing agents was stored at 4 °C for six months and the electrophoresis results are shown in Figure 4. The intensity of the SC form of spray-dried plasmid DNA in Figure 4 decreased as compared with samples without 6-months of ageing (Figures 1, 2). This demonstrated that spray-dried plasmid DNA was further degraded by the ageing process, even when the storage temperature remained as low as 4 °C. The results were also in accordance with a previous observation that

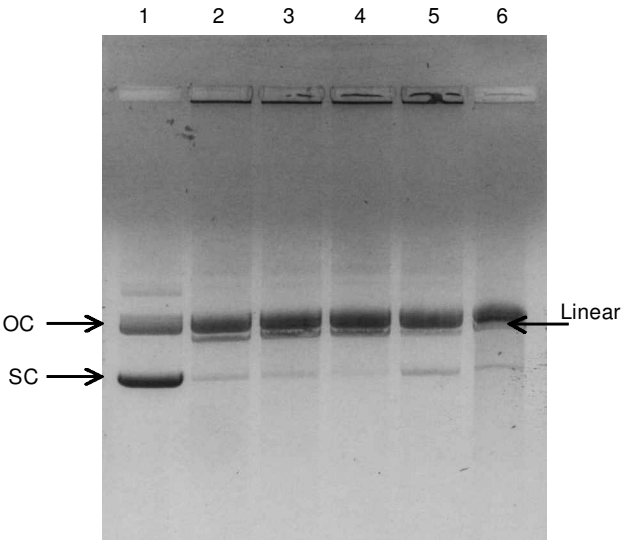

Figure 4 Agarose gel electrophoresis of spray-dried plasmid DNA under the protection of noncondensing agents stored at 4 °C for six months. Lane 1: control plasmid DNA (without spray drying and ageing); lanes 2–4: spray-dried plasmid DNA in 30% sucrose, 5% glycine/20% sucrose, and 2% agarose solutions respectively; lanes 5 and 6: spray-dried plasmid DNA (in 20% sucrose solution) at outlet temperatures of 56 and 69 °C (± 2 °C), respectively. The outlet temperature = 56 ± 2 °C. The band position for supercoiled circular (SC), open circular (OC), and linear DNA is indicated with arrows.

the structural change of plasmid DNA was irreversible; the OC and linear form cannot be converted back to the SC form (Anchordoquy & Koe 2000). As for PEGs (Figure 5, lanes 3 and 4), no linear forms were obtained from the analysis of agarose gel electrophoresis after six months storage at 4 °C, whereas, for PEI–DNA complexes, no band was detected in the agarose gel after spray drying in aqueous solution (Figure 5, lane 5). Apparently, the binding between cationic polymer and plasmid DNA was undisrupted by six months storage at 4 °C. However, after polyaspartic acid dissociation (Figure 5, lanes 2 and 6), plasmid DNA remained in the SC and OC forms in aqueous solution at cationic polymer/DNA weight ratios 5/1 and 3/1. This reflected the stabilizing effects on spray-dried plasmid DNA by the addition of cationic polymers such as PEI. These observations showed clearly that the condensing agents (PEG 1000, PEG 4000, and PEI) were more effective than the noncondensing agents on the long-term stability of plasmid DNA by the process of spray drying. These stability studies have opened the opportunity to using the process of spray drying as a means to facilitate the formulations of plasmid DNA such as aerosols while still preserving plasmid DNA integrity. The protective effect of PEG could be used for storage purposes. However, PEG–DNA complexes are unlikely to be used as aerosols because the charge of the complex would not be suitable for transfection. PEI could be used for transfection in the lung without the use of cationic lipids and could be of paramount interest for the development of commercial gene products.

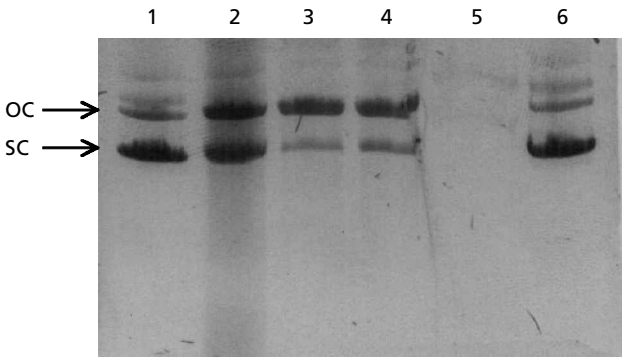

Figure 5 Agarose gel electrophoresis of spray-dried plasmid DNA under the protection of condensing agents stored at 4 °C for six months. Lane 1: control plasmid DNA (without spray drying and ageing); lanes 3 and 4: spray-dried plasmid DNA in 20% PEG 1000 and 20% PEG 4000 solutions, respectively; lane 5: spray-dried plasmid DNA in 20% sucrose solution (PEI/DNA (w/w) 5/1) without polyaspartic acid dissociation; lanes 2 and 6: spray-dried plasmid DNA in 20% sucrose solution (PEI/DNA (w/w) 3/1 and 5/1, respectively) with polyaspartic acid dissociation. The outlet temperature = 56 ± 2 °C. The band position for supercoiled circular (SC) and open circular (OC) DNA is indicated with arrows.

Conclusions

Spray drying had adverse effects on the tertiary structure of plasmid DNA with the addition of noncondensing agents such as sucrose, glycine, and agarose. The damage was evident even at low outlet temperatures during spray drying. Spray-dried plasmid DNA with noncondensing agents was degraded further by the ageing process, despite using 4 °C as the low storage temperature. On the other hand, with the addition of condensing agents such as PEGs and cationic polymers (PEI) the spray-dried plasmid DNA maintained a certain degree of structural integrity. No linear forms of plasmid DNA were revealed in the gel by the protection of these condensing agents. Moreover, DNA topology remained unchanged by such treatments after six-month storage at 4 °C. This study has demonstrated that the DNA condensation utilizing condensing agents may provide a way to minimize structural damage by the process of spray drying.

References

Adam, R. E., Zimm, B. H. (1977) Shear degradation of DNA. *Nucleic Acids Res.* **4**: 1513–1537

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. J., Koe, G. S. (2000) Physical stability of nonviral plasmid-based therapeutics. *J. Pharm. Sci.* **89**: 289–296

Bloomfield, V. A. (1996) DNA condensation. *Curr. Opin. Struct. Biol.* **6**: 334–341

Boussif, O., Lezoualch, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., Behr, J.-P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. USA* **92**: 7297–7301

Broadhead, J., Edmond Rouan, S. K., Hau, I. (1994) The effect of process and formulation variables on the properties of spray-dried β-galactosidase. *J. Pharm. Pharmacol.* **46**: 458–467

Carpenter, J. F., Crowe, J. H. (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* **28**: 3916–3922

Freeman, D. J., Niven, R. W. (1996) The influence of sodium glycocholate and other additives on the in vivo transfection of plasmid DNA in the lungs. *Pharm. Res.* **13**: 202–209

Gebhart, C. L., Kabanov, A. V. (2001) Evaluation of polyplexes as gene transfer agents. *J. Control. Release* **73**: 401–416

Kimoto, H., Taketo, A. (1996) Studies of electrotransfer of DNA into *Escherichia coli*: Effect of molecular form of DNA. *Biochim. Biophys. Acta* **1307**: 325–330

Kuo, J.-H., Lo, Y.-L., Shau, M.-D., Cherng, J.-Y. (2002) A thermodynamic study of cationic polymer-plasmid DNA complexes by high-sensitive differential scanning calorimetry. *J. Control. Release* **81**: 321–325

Labrude, P., Rasolomanana, M., Vigneron, C., Thirion, C., Chaillot, B. (1989) Protective effect of sucrose on spray drying of oxyhemoglobin. *J. Pharm. Sci.* **78**: 223–229

Levy, M. S., Collins, I. J., Yim, S. S., Ward, J. M., Titchener-Hooker, N., Shamlou, P. A., Dunnill, P. (1999) Effect of shear on plasmid DNA in solution. *Bioprocess Eng.* **20**: 7–13

Lindahl, T., Karlstrom, O. (1973) Heat-induced depyrimidination of deoxyribonucleic acid in neutral solution. *Biochemistry* **12**: 5151–5154

Lobo, B. A., Rogers, S. A., Choosakoonkriang, S., Smith, J. G., Koe, G., Middaugh, C. R. (2002) Differential scanning calorimetric studies of the thermal stability of plasmid DNA complexed with cationic lipids and polymers. *J. Pharm. Sci.* **91**: 454–466

Maa, Y.-F., Hsu, C. C. (1997) Protein denaturation by combined effect of shear and air-liquid interface. *Biotechnol. Bioeng.* **54**: 503–512

Maa, Y.-F., Nguyen, P.-A., Andya, J. D., Dasovich, N., Sweeney, T. D., Shire, S. J., Hsu, C. C. (1998) Effect of spray drying and subsequent processing conditions on residual moisture content and physical/biochemical stability of protein inhalation powders. *Pharm. Res.* **15**: 768–775

Master, K. (1991) In: *Spray Drying Handbook*. Fifth edn, Longman Scientific & Technical, London, pp. 1–66

Middaugh, C. R., Evans, R. K., Montgomery, D. L., Casimiro, D. R. (1998) Analysis of plasmid DNA from a pharmaceutical perspective. *J. Pharm. Sci.* **87**: 130–146

Miller, A. D. (1992) Human gene therapy comes of age. *Nature* **357**: 455–460

Niven, R., Ip, A., Mittelmann, S., Farrar, C., Arakawa, T., Prestrelski, S. (1994) Stability of lactate dehydrogenase and recombinant granulocyte colony stimulating factor to air-jet nebulization. *Int. J. Pharm.* **109**: 17–26

Reese, H. R., Zimm, B. H. (1990) Fracture of polymer chains in extensional flow: Experiments with DNA, and a molecular-dynamics simulation. *J. Chem. Phys.* **92**: 2650–2662

Sambrook, J., Russel, D. W. (2001) In: *Molecular Cloning: A Laboratory Manual*. Third edn, Cold Spring Harbor Laboratory Press, New York, Chapter 2, p. 110

Suzuki, T., Ohsumi, S., Makino, K. (1994) Mechanistic studies on depurination and apurinic site chain breakage in oligodeoxyribonucleotides. *Nucleic Acids Res.* **22**: 4997–5003

Trubetskoy, V. S., Loomis, A., Hagstrom, J. E., Budker, V. G., Wolff, J. A. (1999) Layer-by-layer deposition of oppositely charged polyelectrolytes on the surface of condensed DNA particles. *Nucleic Acids Res.* **27**: 3090–3095

Vasilevskaya, V. V., Khokhlov, A. R., Matsuzawa, Y., Yoshikawa, K. (1995) Collapse of single DNA molecule in poly(ethylene glycol) solutions. *J. Chem. Phys.* **102**: 6595–6602

Walsh, G. (1998) In: *Biopharmaceuticals: Biochemistry and Biotechnology*. John Wiley & Sons Ltd, UK, pp. 122–123

Xie, T. D., Tsong, T. Y. (1993) Study of mechanisms of electric field-induced DNA transfection. V. Effects of DNA topology on surface binding, cell uptake, expression, and integration into host chromosomes of DNA in the mammalian cell. *Biophys. J.* **65**: 1684–1689