Preparation and Characterization of Poly (D,L-Lactide-Co-Glycolide) Microspheres for Controlled Release of Poly(L-Lysine) Complexed Plasmid DNA

Yilmaz Capan,[1] Byung H. Woo,[1] Sisay Gebrekidan,[1] Shamim Ahmed,[1] and Patrick P. DeLuca[1,2]

Received November 5, 1998; accepted January 12, 1999

Purpose. To produce and characterize controlled release formulations of plasmid DNA (pDNA) loaded in poly (D,L-lactide-co-glycolide) (PLGA) microspheres both in free form and as a complex with poly (L-lysine).

Methods. Poly (L-lysine) (PLL) was used to form pDNA/PLL complexes with complexation ratio of 1:0.125 and 1:0.333 w/w to enhance the stability of pDNA during microsphere preparation and protect pDNA from nuclease attack. pDNA structure, particle size, zeta potential, drug loading, in vitro release properties, and protection from DNase I were studied.

Results. The microspheres were found to be spherical with average particle size of 3.1–3.5 μm. Drug loading of 0.6% was targeted. Incorporation efficiencies of 35.1% and 29.4–30.6% were obtained for pDNA and pDNA/PLL loaded microspheres respectively. Overall, pDNA release kinetics following the initial burst did not correlate with blank microsphere polymer degradation profile suggesting that pDNA release is convective diffusion controlled. The percentage of supercoiled pDNA in the pDNA and pDNA/PLL loaded microspheres was 16.6 % and 76.7–85.6% respectively. Unencapsulated pDNA and pDNA/PLL degraded completely within 30 minutes upon the addition of DNase I. Encapsulation of DNA/PLL in PLGA microspheres protected pDNA from enzymatic degradation.

Conclusions. The results show that using a novel process, pDNA can be stabilized and encapsulated in PLGA microspheres to protect pDNA from enzymatic degradation.

KEY WORDS: controlled release; microspheres; DNA; DNA/poly(L-lysine) complex; poly(lactide-co-glycolide).

INTRODUCTION

For gene therapy to become a reality, safe and effective methods must be found to deliver DNA efficiently to target cells (1). A number of techniques have been developed for the introduction of genes into cells. Although delivery systems of viral origin, such as retrovirus (2,3) and adenovirus (4), efficiently introduce genes, they suffer from immunogenicity, toxicity, and lack of tissue specificity. The immunogenicity of viral vectors restricts the repeated use of the delivery systems. Non-viral delivery systems, such as cationic lipids, liposomes and polymeric microspheres have been increasingly proposed as alternatives to viral vectors because of potential advantages such as tissue-specific targeting, relative ease of large-scale production, and relative safety (5–7).

Free pDNA is rapidly fragmented within 30 min during in vitro mouse serum incubation due to the action of endonucleases (8). Adami et al. showed that the stabilization afforded by condensation with a peptide protects DNA during formulation and preserves its structure in serum (9). pDNA is also rapidly eliminated from the plasma when injected intravenously into mice without any delivery system (10).

Biodegradable polymers have shown promising results in the delivery of many bioactive peptides (11). The development of biodegradable microspheres for pDNA delivery may offer several advantages over other formulations. First, encapsulation of pDNA in microspheres could protect DNA from rapid in vivo degradation. Secondly, localized delivery of pDNA may increase the amount of pDNA retained within tissues (12).

We are interested in describing a delivery system where PLL complexed pDNA is incorporated in PLGA microspheres. The aim of this study is to determine whether PLL complexed pDNA can be incorporated and continuously released from PLGA microspheres without degradation. The characteristics of the microspheres were examined under various conditions. Characterization included analyzing released pDNA for conformational/structural integrity and release kinetics.

MATERIALS AND METHODS

Materials

pDNA (supercoiled, ~5Kb) was provided by Pangaea Pharmaceuticals Inc. 50:50 poly(D,L-lactide-co-glycolide) (m.w. 32,510, Resomer® RG503, PLGA) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (m.w. 30,000–70,000, PVA) and poly(L-lysine) (m.w. 25,000, PLL) were supplied by Sigma Chemical (St Louis, MO, USA). Pico Green® reagent was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were obtained commercially as analytical grade reagents.

Preparation of Microspheres

Microspheres of PLGA were prepared by modification of a previously described procedure (13). pDNA/PLL complex was prepared by the rapid mixing of 900 μg of pDNA in Tris-EDTA buffer with varying amounts of PLL. In this study two pDNA/PLL complexes (pDNA/PLL ratio, 1:0.125 and 1:0.333, w/w) were used for the preparation of microspheres. Complex formation was allowed to proceed spontaneously at room temperature for 30 min. Experiments were performed to investigate whether PLL forms a complex with pDNA. Physicochemical characterization of the pDNA/PLL complex was examined by PicoGreen® dye exclusion assay and agarose gel electrophoresis with ethidium bromide staining as described below. The complex was prepared immediately prior to the experiments. The microspheres were prepared by dispersing an aqueous solution of pDNA or pDNA/PLL complex into a 6% (w/w) solution of PLGA dissolved in methylene chloride followed by 2min. vortex mixing. The primary w/o dispersion was injected with a syringe into an aqueous 4% PVA solution containing 10% sucrose (continuous phase) at 15°C while being mixed with a

[1] University of Kentucky, College of Pharmacy, Faculty of Pharmaceutical Sciences, Rose Street, Lexington, Kentucky 40536.

[2] To whom correspondences should be addressed. (e-mail: ppdelu1@pop.uky.edu)

0724-8741/99/0400-0509$16.00/0

Silverson laboratory mixer-L4R (Silverson Machines Inc, USA.). The solvent was extracted by transferring the resulting w/o/w emulsion into 150 ml of 0.35% PVA solution containing 10% sucrose and stirring for 1 hr at 37°C. The solidified microspheres were recovered by filtration and dried under vacuum at room temperature.

Particle Characterization

Particle Size Distribution

Particles were sized by laser diffractometry using a Malvern 2600 laser sizer (Malvern Instruments PC6300, England). The average particle size was expressed as the volume mean diameter v_{md} in μm.

Surface Morphology

The surface morphology was examined by scanning electron microscopy (Hitachi Model S800, Japan) after palladium/gold coating of the microsphere sample on an aluminum stub.

Zeta Potential Measurement

Zeta potential of microspheres was measured in 10 mM Tris-HCl buffer (pH 7.4) using a Zeta Meter (Zeta Meter, Inc.) equipped with a Nikon SMZ-2 stereoscopic microscope. Velocity measurements were performed on 10 individual particles at a voltage of 150 V and the zeta potential was calculated using the average velocity of 5 runs.

Determination pDNA Loading of Microspheres

PLGA microspheres were dissolved in chloroform. pDNA or pDNA/PLL complex was extracted from the polymer solution by addition of 10 mM Tris-EDTA buffer (pH 7.4). The amount and conformation of the extracted pDNA, or pDNA/PLL complex was analyzed to determine encapsulation efficiency and potential alterations in plasmid structure. The quantification and conformational analysis of the pDNA and the pDNA/PLL complex will be reported elsewhere (unpublished data). Briefly, pDNA loading was determined by Fluorescence spectrophotometry (H-2000 Hitachi, Japan) using Picogreen® reagent. pDNA stability and topology were assessed by 0.8% agarose gel electrophoresis (Pharmacia Fine Chemicals, Sweden) using 40 mM Tris-acetate buffer containing 1 mM EDTA (TAE) and ethidium bromide staining. The ratio of supercoiled to degraded linear pDNA was quantitated densitometrically using a Kodak Scanner (Kodak digital science, Electrophoresis Documentation and Analysis System 120).

In Vitro Release Study

The release experiments were carried out in 33 mM phosphate buffer (pH 7.4). 10 milligrams of microspheres were added to 10 mL of phosphate buffer in 15 mL conical centrifuge tubes incubated at 37°C. At predetermined time intervals, 1 mL aliquots of the supernatant were removed. The dissolution medium was replaced with fresh buffer after each sampling. The concentration of pDNA in the supernatant was determined by fluorescence spectrophotometry using the Picogreen® dye assay. Released pDNA or pDNA/PLL complex was analyzed by agarose gel electrophoresis and compared to unencapsulated stock pDNA or pDNA/PLL complex to determine the conformation of released pDNA.

Mass Loss Study

50 milligrams of blank PLGA microspheres were added to 50 mL of 33 mM phosphate buffer (pH 7.4) in 50 ml conical centrifuge tubes incubated at 37°C. At different time intervals, the tubes were removed and the microspheres were collected by filtration (0.22 μm, Millipore). The microspheres were vacuum dried and weighed.

DNase I Digestion Study

pDNA, pDNA/PLL complexes and pDNA microspheres were incubated with 5 μg of DNase I in 10 mM Tris-HCl buffer containing 10 mM $MgSO_4$ (pH 8.0) for 30 min at 37°C. Following digestion, samples were analyzed by 0.8% agarose gel electrophoresis for DNA fragments. Kodak scanning was used to quantitate the ratio of supercoiled to linear pDNA.

RESULTS AND DISCUSSION

Preparation of pDNA/PLL Complex

Picogreen® dye exclusion proved to be a useful indicator of complexation between PLL and pDNA. As PLL was added to pDNA, the fluorescence emitted decreased until a minimum level was reached close to baseline fluorescence. PLL was shown to form a complex with pDNA where up to 95% of the original fluorescence intensity was quenched. Gel retardation assay showed that the movement of pDNA was retarded as the amount of PLL increased indicating that PLL forms a complex with pDNA (data not shown). The pDNA/PLL complexes show much weaker bands in intensity, probably due to the exclusion of ethidium bromide following formation of complexes. When increasing amounts of PLL were added to the pDNA, the pDNA band completely disappeared suggesting that the complexes formed were too large to migrate into the gel matrix or that pDNA was completely covered by PLL thereby preventing ethidium bromide staining.

Effect of Processing on Microsphere Size and DNA Entrapment

The process of microsphere preparation by the solvent evaporation method, using a double emulsion, involves several steps that can damage pDNA. Exposure to high interfacial tension at the methylene chloride/water interphase, ultrasonic radiation and vortex mixing during preparation of the primary w/o dispersion affect the stability of pDNA. Preliminary studies to examine the effect of each step in the preparation process on the total amount and the stability of pDNA showed that sonication using a probe sonicator (Heat Systems, Ultrasonics, Inc.) for 60 s, to form the primary w/o dispersion, has the most detrimental effect on pDNA stability. Following sonication, pDNA was completely degraded in the primary w/o dispersion (data not shown). Therefore, sonication was avoided in the preparation of the dispersed phase and vortex mixing was adopted. The addition of 10% sucrose to the PVA solution

increased pDNA stability. Preliminary work indicated that maximum loading efficiency was obtained when a 4% PVA solution was used for the production of pDNA microspheres. This result contrasts with a recent study, in which, the PVA concentration had no significant effect on drug encapsulation (14).

PLGA microspheres were prepared from pDNA and pDNA/PLL complexes. The corresponding preparation conditions, loading efficiency, yield and average particle sizes for the various batches (F1 - F3) are summarized in Table 1. Actual drug loading efficiencies ranged between 29.4 ± 0.9 and 35.1 ± 5.6%. The average yield was 68 ± 2.0–72 ± 1%. Higher drug loading was achieved in the formulation when pDNA alone was used. However, only minor differences in drug loading were observed between batches prepared from pDNA/PLL complexes. The scanning electron micrographs shown in Fig. 1 reveal spherical particles with particle size in the range of 3.1–3.5 μm.

Zeta Potential

Surface charge of the pDNA delivery system is expected to influence its interactions with various biological components, as well as its distribution, access and entry into target cells. pDNA loaded microsphere exhibited a relatively high negative surface charge (Fig. 2). As expected, complexation of pDNA with PLL led to a decrease in the negative surface charge of the microspheres. Further increase in PLL resulted in surface charge that was similar to the zeta potential of blank microspheres. This observation was consistent with published literature on PLL based systems (15).

In Vitro Release

Release kinetics experiments were carried out in 33 mM phosphate buffer (pH 7.4) at 37°C. Previous studies had shown that pDNA and pDNA/PLL complex are stable in phosphate buffer pH 7.4 (unpublished data). pDNA and pDNA/PLL (1:0.333, w/w) loaded microspheres exhibited a high burst effect with 34% drug release after one day compared to the pDNA/PLL (1:0.125, w/w) loaded microspheres (Fig. 3). In general, the release of drugs from PLGA microspheres occurs by two mechanisms. The first is diffusion of drug molecules through aqueous pores or channels formed during microsphere preparation or after the dissolution and release of surface localized drug. The strong burst effect observed for free pDNA and pDNA/PLL loaded microspheres, is probably due to the diffusion of surface localized drug. The second mechanism involves the degradation and solubilization of the PLGA matrix. The drug release profiles show that 90%, 83% and 72% of the drug is released within 20 days from pDNA, pDNA/PLL (1:0.333, w/w) and pDNA/PLL (1:0.125, w/w) loaded microspheres respectively. However, there is no significant mass loss of PLGA matrix until 30 days (Fig. 3). Therefore, convective diffusion through porous matrix is proposed as the mechanism of release of pDNA from the microspheres.

Analysis of pDNA Structural Integrity

The development of optimized DNA microspheres for gene therapy requires an understanding of DNA stability during formulation and during transport to the target site. The conversion of DNA from supercoiled to circular only has a minor effect on the gene transfer efficiency. However, subsequent conversion to linear DNA reduces gene expression by 90%, whereas transformation to oligonucleotide fragments via sonication reduced gene expression nearly 1000-fold (9).

The stability profile of DNA loaded microsphere formulations has not been reported previously. In the present study, the influence of preparation process on the stability of supercoiled pDNA was examined. As shown in Fig. 4, pDNA was present in the microspheres in the supercoiled and linear conformations. A significant drop in percentage supercoiled content was observed for the uncomplexed pDNA loaded microspheres. There was extensive smearing in the gel electrophoresis of the pDNA loaded microspheres compared to pDNA/PLL loaded microspheres indicating that supercoiled pDNA was degraded in the uncomplexed pDNA loaded microspheres.

The extraction process was validated to ensure that the process did not degrade pDNA (unpublished data). The preparation process for uncomplexed pDNA loaded microspheres resulted in the conversion of most of the supercoiled pDNA to the linear structure. Complexation of pDNA with PLL increases the stability of supercoiled form in the microspheres (Table 1). The percentage of supercoiled pDNA in the microspheres was only 16.6% for the uncomplexed pDNA but increased to 76.7% and 85.6% upon 1:0.125 (w/w) and 1:0.333 (w/w) complexation with PLL, respectively.

DNase I Treatment

The endonuclease activity of DNase I results in the fragmentation of unprotected pDNA. Microencapsulation of pDNA is expected to protect pDNA from enzymatic degradation. In this study, free solutions of pDNA, pDNA/PLL complex and microencapsulated free pDNA and pDNA/PLL were each incubated with DNase I. Free solution of pDNA was completely fragmented within 30 min. Incubation of pDNA/PLL with DNase I resulted in a degradation profile that was similar to

Table 1. Effect of pDNA/PPL Complexation Ratio and DNase I Digestion on the pDNA and pDNA/PLL Complex Loaded Microspheres

Formulation	pDNA load (%)	pDNA:PPL ratio (w/w)	Loading efficiency (%)	Particle size (μm)	Yield (%)	DNase I treatment		
						Recovery (%)	Mean % supercoiled	
							Before	After
F1	0.6	1:0	35.1 ± 5.6	3.5 ± 1.6	68 ± 2	61.5 ± 2.1	16.6	12.4
F2	0.6	1:0.125	30.6 ± 1.6	3.3 ± 0.2	72 ± 1	96.5 ± 1.6	76.7	71.9
F3	0.6	1:0.333	29.4 ± 0.9	3.1 ± 0.1	71 ± 2	97.9 ± 2.0	85.6	84.1

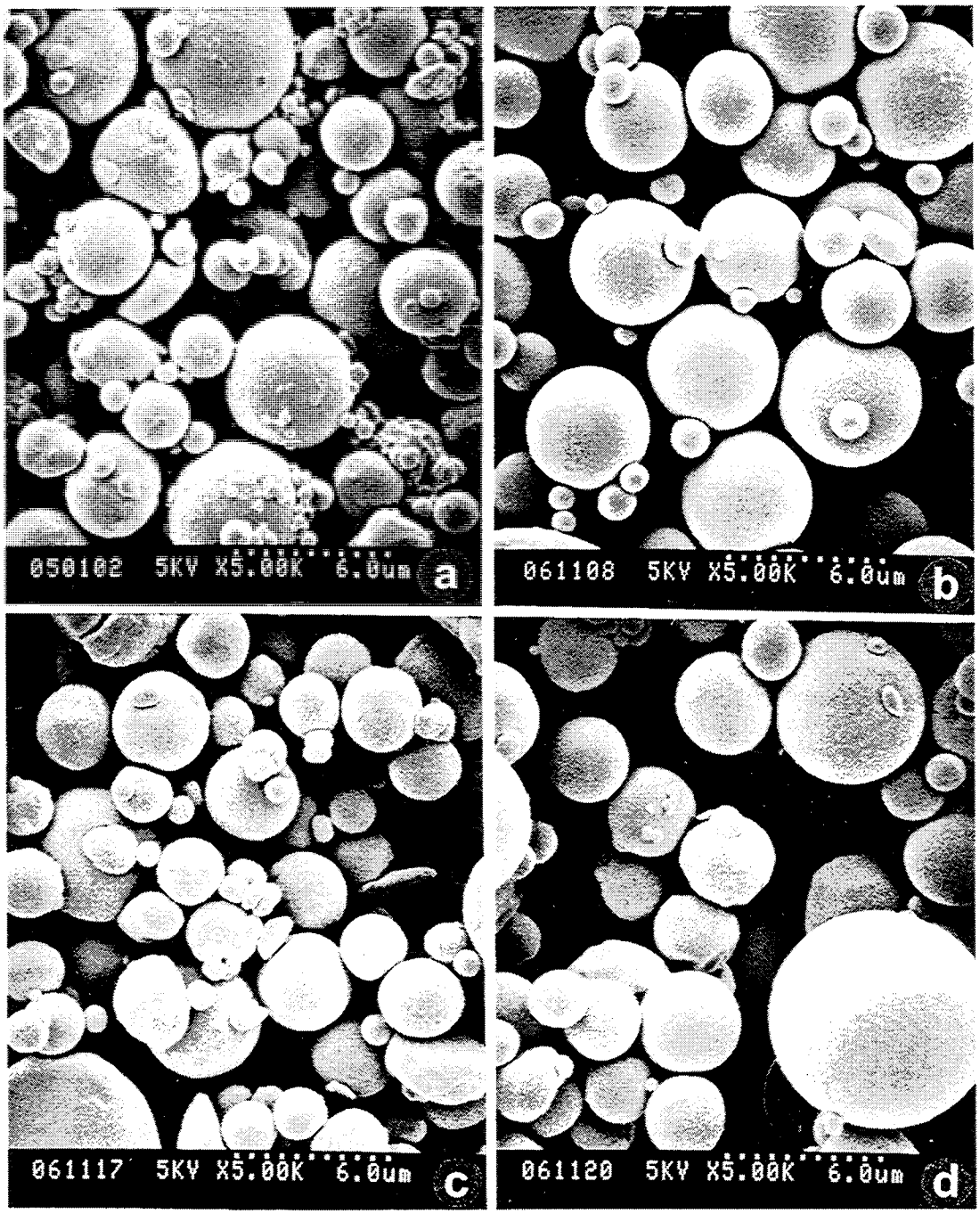

Fig. 1. SEM micrographs of a) blank, b) pDNA, c) pDNA/PLL (1:0.125 w/w), d) pDNA/PLL (1:0.333, w/w) loaded microspheres.

that observed for free solution of pDNA (data not shown). Table 1 shows that microencapsulation of pDNA and pDNA/PLL had a profound effect on protecting pDNA from enzymatic degradation. Percentage of total DNA recovery after enzyme treatment increased from 61.5 ± 2.1 for free pDNA loaded microspheres to 96.5 ± 1.6 and 97.9 ± 2.0 for 1:0.125 and 1:0.333 (w/w) pDNA/PLL loaded microspheres, respectively. Complexation of pDNA with PLL before microencapsulation appears to increase the stability of pDNA. The supercoiled structure of pDNA was maintained after DNase I treatment in the pDNA/PLL-loaded microspheres. Prior to enzyme treatment, 76.7% of the pDNA was in the supercoiled conformation in the pDNA/PLL (1:0.125, w/w) loaded microspheres. After enzyme treatment 71.9% of pDNA remained in the supercoiled conformation. Similarly, the percentage of supercoiled pDNA only changed from 85.6% to 84.1% after DNase I treatment for the pDNA/PLL (1:0.333, w/w) loaded microspheres (Table 1).

CONCLUSIONS

The *in vivo* delivery of DNA remains a challenge because DNA is rapidly recognized and degraded by nucleases in the plasma leading to half-life of few minutes. In addition, the high negative charge of pDNA prevents transport across cellular

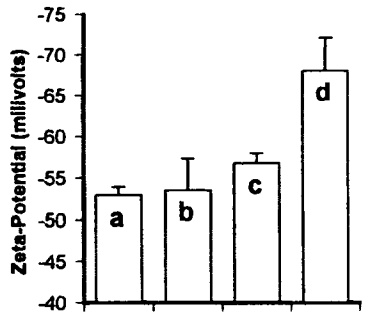

Fig. 2. Zeta potential of microspheres, a) pDNA/PLL (1:0.333, w/w), b) blank, c) pDNA/PLL (1:0.125, w/w), d) pDNA loaded microspheres.

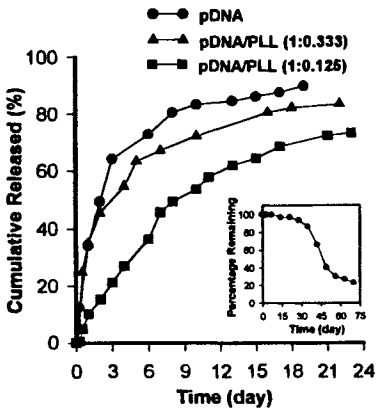

Fig. 3. Cumulative release of pDNA and pDNA/PLL from microspheres (large panel) and mass loss of blank microspheres (small panel).

membrane. A delivery system for pDNA has been developed that encapsulates poly(L-lysine) complexed pDNA in poly(lactide-co-glycolide) microspheres. The encapsulation of pDNA in microspheres protects pDNA from nucleases. PLL complexation with pDNA was found to increase the stability of pDNA in the microspheres. PLGA microspheres are biodegradable, biocompatible and may potentially be useful for the *in vivo* delivery of DNA. Future studies will focus on evaluating the *in vitro* and *in vivo* transfection efficiency of this delivery system.

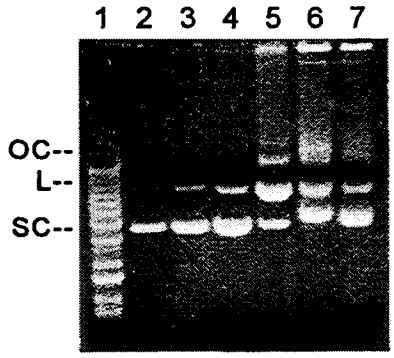

Fig. 4. Agarose gel electrophoresis for the assessment of pDNA integrity. Lane 1) molecular weight marker, Lanes 2-4) 0.2; 0.5; 1 μg pDNA, lane 5) pDNA loaded, lanes 6,7) pDNA/PLL (1:0.125, w/w), and pDNA/PLL (1:0.333, w/w) loaded microspheres.

ACKNOWLEDGMENTS

The authors thank Pangaea Pharmaceuticals Inc. for the supply of pDNA and Professor Kenneth B. Ain and Mustafa Yatin, Ph. D. UK, Thyroid Cancer Research Laboratory, Department of Internal Medicine, Veterans Affairs, Medical Center-Lexington, KY 40536 for performing the Kodak Scanning studies.

REFERENCES

1. R. J. McDonald, H. D. Liggitt, L. Roche, H. T. Nguyen, R. Pearlman, O. G. Raabe, L. B. Bussey, and C. M. Gorman. Aerosol Delivery of lipid:DNA complexes to lungs of Rhesus monkeys. *Pharm. Res.* **15**:671–678 (1998).
2. A. Ido, K. Nakata, Y. Kato, K. Nakao, K. Murata, M. Fujita, N. Ishii, T. Tamaoki, H. Shiku, and S. Nagataki. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human α-fetoprotein gene promotor. *Cancer Res.* **55**:3105–3109 (1995).
3. G. S. Harrison, C. J. Long, T. J. Curiel, F. Maxwell, and I. H. Maxwell. Inhibition of human immunodeficiency virus-1: Production resulting from transduction with a retrovirus containing an HIV-regulated diphtheria toxin A chain gene. *Hum. Gene Ther.* **3**:461–469 (1992).
4. J. M. Friedman, L. E. Babiss, D. F. Clayton, and J. E. Darnell. Cellular promoters incorporated into the adenovirus genome: cell specificity of albumin and immunoglobulin expression. *Mol. Cell. Biol.* **6**:3791–3797 (1986).
5. M. Cotten and E. Wagner. Non-viral approaches to gene therapy. *Curr. Opin. Biotechnol.* **4**:705–710 (1993).
6. R. I. Mahato, A. Rolland, and E. Tomlinson. Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharm. Res.* **14**:853–859 (1997).
7. D. H. Jones, S. Corris, S. McDonald, J. C. S. Clegg, and G. H. Farrar. Poly (D,L-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* **15**:814–817 (1997).
8. K. Kawabata, Y. Takakura, and M. Hashida. The fate of plasmid DNA after intravenous injection in mice. Involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* **12**:825–830 (1995).
9. R. C. Adami, W. T. Collard, S. A. Gupta, K. Y. Kwok, J. Bonadio, and K. G. Rice. Stability of peptide-condensed plasmid DNA formulations. *J. Pharm. Sci.* **87**:678–683 (1998).
10. M. Yoshida, R. I. Mahato, K. Kawabata, Y. Takakura, and M. Hashida. Disposition characteristics of plasmid DNA in the single-pass rat liver perfusion system. *Pharm. Res.* **13**:599–603 (1996).
11. R. C. Mehta, R. Jeyanthi, S. Calis, B. C. Thannoo, K. W. Burton, and P. P. DeLuca. Biodegradable microspheres as depot system for parenteral delivery of peptide drugs. *J. Contr. Rel.* **29**:375–384 (1994).
12. H. L. Davis, R. G. Whalen, and B. A. Demeneix. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum. Gene. Ther.* **4**:733–740 (1993).
13. R. Jeyanthi, B. C. Thanoo, R. C. Mehta, and P. P. DeLuca. Effect of solvent removal technique on the matrix characteristics of polylactide/glycolide microspsheres for peptide delivery. *J. Contr. Rel.* **38**:235–244 (1996).
14. J. Hermann and R. Bodmeier. Somatostatin containing biodegradable microspheres prepared by a modified solvent evaporation method based on w/o/w-multiple emulsions. *Int. J. Pharm.* **126**:129–138 (1995).
15. C. W. Pouton, P. Lucas, B. J. Thomas, A. N. Uduehi, D. A. Milray, and S. H. Moss. Polycation-DNA complex for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *J. Contr. Rel.* **53**:289–299 (1998).