# PLGA Microspheres Containing Plasmid DNA: Preservation of Supercoiled DNA via Cryopreparation and Carbohydrate Stabilization

SHUICHI ANDO, DAVID PUTNAM, DANIEL W. PACK, AND ROBERT LANGER*

Contribution from *Massachusetts Institute of Technology, E25-342, 45 Carleton Street, Cambridge, Massachusetts 02139.*

**Abstract** □ Biodegradable microspheres containing plasmid DNA have potential uses as mediators of transfection in cells, particularly phagocytic cells such as macrophages. However, the hydrophilic nature and the structural instability of supercoiled DNA preclude its facile encapsulation in polymer matrixes such as poly(*d,l*-lactic-*co*-glycolic acid) (PLGA) by traditional methods. We initially studied the microencapsulation of plasmid DNA using the established water-in-oil-in-water double-emulsion solvent-evaporation method and found that (1) the encapsulation efficiency was low (about 20%), (2) the microencapsulation procedure nicked (degraded) the supercoiled DNA, and (3) lyophilization of the microsphere also nicked the DNA. We have therefore designed a new microsphere preparation method (called cryopreparation) to specifically address these concerns. Using the cryopreparation method, the aqueous phase of the primary emulsion containing the plasmid DNA is frozen and then subjected to homogenization. Because there is no shear stress inside a solid, we hypothesized that freezing the aqueous phase of the primary emulsion would help to preserve the supercoiled plasmid DNA during formation of the secondary emulsion. We also hypothesized that the formation of crystals from buffers within the primary emulsion was a causative factor for nicking during freezing or lyophilization, and that disruption of the crystal formation by the addition of saccharides into the primary emulsion would improve the supercoiled-DNA content of the spheres. Our results support the two hypotheses. Not only was the supercoiled-DNA content increased from 39% to over 85%, but the encapsulation efficiency was also elevated from 23% to over 85%.

## Introduction

The importance of DNA therapeutics, in particular in gene therapy, has led to increased research and development in this area.[1-5] The use of such therapeutics can be problematic, however, because during delivery the DNA is subject to degradation. To maximize the power of these agents, it would be desirable to develop a mode of delivery in which the DNA-based therapeutic is protected from degradation. It is known that nano- or micro-encapsulation techniques can be used to protect sensitive bioactive agents, such as DNA, from degradation.[6-10]

Biodegradable microspheres, such as those based on PLGA, have the potential to act as mediators of DNA transfection targeted to phagocytic cells such as macrophages, and to protect against biological degradation by nucleases. Uptake of biodegradable microspheres by macrophages has been extensively studied,[11] and more recently Ciftci et al.[12] reported a method to introduce DNA into mammalian cells using a polymer-based gene-delivery system. Spheres in the range of $1-10~\mu m$ are too large to enter cells via endocytosis, and therefore "target" phago-cytic cells such as macrophages by size exclusion. Because macrophages are antigen-presenting cells for the immune system, microencapsulated plasmid DNA is particularly useful for the rapidly developing field of DNA-based vaccines.[13]

One of the most common techniques for preparation of biodegradable polymer microspheres encapsulating hydrophilic molecules is the double-emulsion solvent-evaporation method. Using this technique, the molecule to be encapsulated is placed in aqueous solution while the polymer is dissolved in an organic phase commonly consisting of methylene chloride or ethyl acetate. The two phases (volume organic/volume aqueous = $3-20$) are emulsified, typically by sonication or homogenization. This primary emulsion is then added to a second aqueous phase (20- to 100-fold larger volume) and again mixed by homogenization to form the (water-in-oil)-in-water double emulsion. Upon evaporation of the partially water-miscible solvent, the polymer-containing droplets harden to form microspheres which can then be isolated by filtration or centrifugation. Lyophilization removes water from the interior aqueous phase resulting in a dry suspension of the encapsulated material within the polymer matrix.

Our initial experiments using the standard double-emulsion microsphere formulation process demonstrated the tendency of plasmid DNA to be converted from its supercoiled state to a nicked or linear state. The preservation of the supercoiled DNA is important because it is known that supercoiled DNA retains the highest level of bioactivity.[14-16] Furthermore, the encapsulation efficiency of DNA into the hydrophobic matrix of PLGA was low, on the order of 20%. On the basis of these observations, we formulated hypotheses for the mechanisms of DNA degradation during the microsphere formulation process and then adopted a design approach to develop an encapsulation process that maximized both the supercoiled-DNA content in the microsphere and its overall encapsulation efficiency. Our hypotheses were as follows: (1) shear stress-induced plasmid DNA degradation occurred during homogenization and (2) buffer salt crystal formation induced plasmid DNA degradation during freezing or drying.

The first hypothesis led to the design and development of a new microencapsulation process for plasmid DNA called cryopreparation. Cryopreparation describes a process in which the temperature of the DNA-containing primary emulsion is lowered below the freezing point of the aqueous inner phase resulting in a solid particulate suspension prior to homogenization to form the secondary emulsion. Because the shear stress within a solid equals zero, the plasmid DNA frozen in the inner phase is exposed to minimum shear stress during homogenization, and the supercoiled state of the plasmid DNA should be preserved. Cryopreparation should also enhance the overall encapsulation efficiency by preventing diffusion of the plasmid DNA out of the microsphere during homogenization. Cryopreparation differs from other microsphere preparation methods that

* To whom correspondence should be addressed. Phone: (617) 253-3123. Fax: (617) 258-8827. E-mail: rlanger@mit.edu.

use temperature as a variable parameter in that cryo-preparation involves selective freezing of the aqueous inner emulsion whereas other procedures freeze or gel the entire microsphere.[17,18] The second hypothesis led to the inclusion of saccharides in the primary emulsion to disrupt the formation of DNA-nicking crystals during homogenization and lyophilization.

## Experimental Section

**Materials**—Plasmid DNA (pCMV-$\beta$-gal) was purified from *E. coli* (DH5$\alpha$) using Plasmid Mega Kit column isolation (QIAGEN, CA), followed by ethanol precipitation. Poly(*d,l*-lactic-*co*-glycolic acid) (PLGA), with a comonomer ratio of 50:50 and an inherent viscosity $\eta = 0.4$ (Resomer RG503, MW 34 000) was purchased from Boehringer Ingelheim (Germany). The emulsifier, poly(vinyl alcohol) (PVA) (88 mol % hydrolyzed, MW 25 000), was purchased from Polysciences, Inc. (Warrington, PA). All other chemicals used were of the highest grade commercially available.

**Cryopreparation**—DNA-containing microspheres were prepared following the established water-in-oil-in-water double-emulsion solvent-evaporation method[19] except for specific steps noted below. The two phases, consisting of 250 $\mu$L of DNA solution (250 $\mu$g of DNA) and 7 mL of methylene chloride containing 200 mg of PLGA, were emulsified by sonication for 10 s (ultrasonic probe, Sonic & Materials Inc.) at room temperature. The primary emulsion temperature was then lowered below the freezing point of the aqueous inner phase by liquid nitrogen immersion, and 50 mL of a 5% PVA solution (4−7 °C) was added and homogenized at 5000−9000 rpm for 14 s (Silverson L4R homogenizer). After homogenization, the resulting emulsion was diluted in 100 mL of 1% PVA, and the system was stirred magnetically for 3 h to allow for evaporation of the organic solvent. Microspheres were finally collected by centrifugation and washed 3 times with water to remove excess PVA. Note that all PVA solutions were adjusted to the osmotic pressure of the inner aqueous phase using agents such as saccharides. The microspheres were resuspended in approximately 1 mL of water, frozen in liquid nitrogen, and lyophilized at room temperature for 24 h on a Labconco Freeze-Dryer 8.

**Achieving DNA Stability against Lyophilization Using Excipients**—The effect of lyophilization on DNA was studied by directly lyophilizing DNA samples (20 $\mu$g/mL). Aliquots (1 mL each) of the DNA solutions, with or without excipients, were frozen in 20-mL disposable scintillation vials by liquid nitrogen immersion and immediately lyophilized at room temperature for 15 h.

**Optimized Microsphere Preparation**—The two phases, consisting of 250 $\mu$L of DNA in water (750 $\mu$g of DNA) containing 1 mM EDTA and 300 mM lactose (pH 7.0) and 7 mL of methylene chloride containing 200 mg of PLGA, were emulsified by sonication as described above. After the primary emulsion was cryoprepared, 50 mL of 5% PVA solution containing 300 mM lactose was added to the solution and homogenized at 7000 rpm for 14 s. The resulting double emulsion was then diluted in 100 mL of 1% PVA solution with 300 mM lactose, and the system was stirred magnetically for 3 h to allow the evaporation of the organic solvent. Microspheres were finally collected by centrifugation, washed three times with distilled water, and then lyophilized at room temperature to obtain a powder.

**Analysis of DNA Structure**—The DNA structure was analyzed by agarose gel electrophoresis (1% agarose containing ethidium bromide, 110 V, 90 min) and compared to untreated stock DNA for semiquantitative determination of supercoiled-DNA content using a Bio Rad Gel Doc 1000 (Software, Molecular Analyst 2.1).

As shown in Figure 1, each DNA band was integrated as a volume. Supercoiled-DNA (SC DNA) content was defined as the volume integrated from the supercoiled DNA band over the total volume integrated from all bands. In other words, SC DNA content equals (SC DNA volume − background volume)/{SC DNA volume + linear DNA volume + nicked DNA volume − (3 × background volume)}. SC DNA content remaining after preparation was calculated as follows: SC DNA remaining (initial %) = 100 × (SC DNA content of sample DNA)/(SC DNA content of initial DNA).

**Quantitation of DNA Content in Microsphere**—The microsphere DNA content was determined using fluorescent reagent PicoGreen (Molecular Probes, Eugene, OR) which preferentially
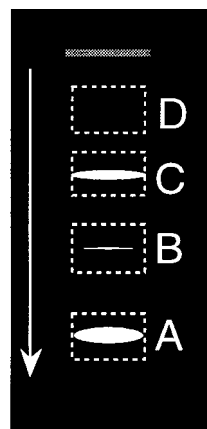


**Figure 1**—Schematic representation of agarose gel electrophoresis of DNA: (A) supercoiled DNA, (B) linear DNA, (C) nicked DNA, and (D) background.

**Table 1**—Effect of Homogenization Rate, Cryopreparation, and Addition of EDTA on Remaining Supercoiled DNA (SC DNA) ($N = 3$)

| DNA solution | SC DNA remaining (initial% ± SD) at homogenization rate | | |
| --- | --- | --- | --- |
| | 5000 rpm | 7000 rpm | 9000 rpm |
| water without cryopreparation | 36.9 ± 1.9 | 26.1 ± 3.6 | 20.1 ± 3.4 |
| water with cryopreparation | 54.4 ± 1.1 | 46.2 ± 0.7 | 37.3 ± 4.7 |
| EDTA[a] without cryopreparation | 68.4 ± 1.9 | 47.0 ± 2.6 | 40.6 ± 0.3 |
| EDTAa with cryopreparation | 92.2 ± 1.3 | 75.5 ± 2.1 | 64.7 ± 3.5 |

[a] 1 mM EDTA, pH 7.5.

binds to double stranded DNA and to a lesser extent to single stranded DNA. Fluorescence ($\lambda_{ex} = 480$ nm, $\lambda_{em} = 520$ nm) of extracted DNA was compared to a standard curve, using plasmid DNA, which was linear from 1 to 50 ng/mL. The encapsulation efficiency was determined as the amount of DNA recovered from the microspheres relative to the initial amount of DNA used (encapsulation efficiency = 100 × (DNA recovered)/(initial DNA)).

**Particle Size of Microsphere**—Particle size distribution of microspheres was analyzed by a Coulter MultisizerII (Coulter Electronics Inc., Hialeah, FL), and the mean volume diameter distribution was determined.

## Results and Discussion

**Stability of DNA Structure against Shear Stress**— In the cryopreparation method, the aqueous phase of the primary emulsion is frozen. Since the shear stress inside a solid equals zero, we hypothesized that cryopreparation would help to preserve the supercoiled DNA during homogenization. To test this hypothesis, the structure of the DNA after homogenization (and before lyophilization) was checked by agarose gel electrophoresis. Table 1 indicates that the supercoiled-DNA content decreases with an increase in homogenization rate, and that using the cryopreparation method preserves the supercoiled-DNA content. These results support our hypothesis that freezing the inner DNA solution protects the DNA from degradation by shear stress, and suggests that cryopreparation is a useful method to prevent supercoiled DNA from degrading during microsphere preparation. When ethylenediaminetetraacetic acid (EDTA) was added to the DNA solution, the supercoiled-DNA content of the resulting microspheres was significantly higher than in the absence of EDTA. EDTA is a chelator of divalent metal cations and inhibits the activity of DNase by this mechanism.[16] Therefore, it was possible that the DNA stability in the presence of EDTA was actually due to DNase inhibition. To examine the possibility of calcium-dependent DNase-mediated degradation, we also included a calcium ionophore ($N,N,N,N$-

**Table 2—Effect of Excipients on Retaining Supercoiled DNA (SC DNA)[a]**

| DNA solution | SC DNA remaining (initial% ± SD) |
|---|---|
| water | 46.2 ± 0.7 |
| 1 mM EDTA solution, pH 7.5 | 75.5 ± 2.1 |
| 1 mM calcium ionophore II | 36.1 ± 0.8 |
| 10 mM Tris buffer, pH 7.5 | 40.5 ± 2.5 |
| phosphate-buffered saline, pH 7.5 | 36.1 ± 3.5 |
| 300 mM lactose solution, pH 7.5 | 39.6 ± 2.7 |
| 1 mM EDTA/10 mM Tris buffer, pH 7.5 | 62.1 ± 2.7 |
| 1 mM EDTA/phosphate-buffered saline, pH 7.5 | 68.2 ± 5.2 |
| 300 mM lactose/1 mM EDTA solution, pH 7.5 | 94.5 ± 1.0 |
| 300 mM lactose/1 mM EDTA/10 mM Tris buffer, pH 7.5 | 86.2 ± 3.6 |

[a] Samples were prepared by cryopreparation; homogenization rate = 7000 rpm, 14 s ($n = 3$).

tetracyclohexyldiglycolic diamide: calcium ionophore II, log $K_{Ca} = 10.1$, $\mu = 0.1$, 20 °C)[20] instead of EDTA (log $K_{Ca} = 11.0$, $\mu = 0.1$, 20 °C).[21] As shown in Table 2, it is apparent that the calcium ionophore II did not act as a DNA stabilizer. Therefore, it is likely that the mechanism of DNA stabilization during cryopreparation is not a result of calcium-dependent DNase inhibition. However, DNase activity dependent on other divalent ions such as manganese and magnesium,[22] could not be eliminated. In addition, Table 2 indicates that DNA degradation during cryopreparation was not inhibited by the addition of PBS (1 mM $K_2HPO_4$, 10 mM $Na_2HPO_4$, 137 mM NaCl, 2.7 mM KCl, pH 7.0), Tris, or lactose to the DNA solution. In the case of addition of PBS or Tris to the DNA solution containing unbuffered EDTA, the supercoiled-DNA content was slightly lower than that in unbuffered EDTA solution, but we found that by adding lactose to the DNA/EDTA solution, the supercoiled-DNA content was increased from 75% to 95%. The exact mechanism of DNA stabilization is unknown. However, it is apparent that the presence of both lactose and EDTA in the DNA solution is important for the stabilization of supercoiled DNA against degradation during cryopreparation.

Regarding the analysis of the DNA structure by agarose gel electrophoresis, it is well-known that the intercalation efficiency of ethidium bromide to DNA is not the same among supercoiled, nicked, and linear DNA. In fact, the intercalation efficiency of ethidium bromide to supercoiled DNA is the lowest.[23] Thus we consider that the estimation of SC DNA ratio in our method is lower than the true value, and that this analytical method is a semiquantitative determination of the supercoiled-DNA content.

Predictably, the microsphere diameters are dependent on the homogenization rate.[24] The volume-mean diameters of microspheres formulated by cryopreparation were 10.2 (±2.4) $\mu$m at 5000 rpm, 4.6 (±0.3) $\mu$m at 7000 rpm, and 3.6 (±0.2) $\mu$m at 9000 rpm ($N = 3$). For optimum phagocytosis by macrophages, spheres with a size of approximately 5 $\mu$m or less are desired.[11] However, increasing the homogenization rate leads to increased DNA degradation. Therefore, homogenization at 7000 rpm was optimal for the cryopreparation method.

**Stability of DNA Structure during Lyophilization**—DNA samples were directly lyophilized to study the effect of lyophilization on DNA stability. Table 3 indicates that the DNA stability in 300 mM lactose and in 1 mM EDTA was the same as that of DNA in water. On the other hand, when Tris buffer or PBS was used in the DNA solution, DNA degradation was increased. Salts such as sodium phosphate are known to form crystals upon freezing,[25] and we speculated that DNA nicking was caused by the salt crystallization. (In the presence of 1 mM EDTA, however, the salt concentration might not be high enough

**Table 3—Effect of Lyophilization on Retaining Supercoiled DNA (SC DNA)[a]**

| DNA solution | SC DNA remaining (initial% ± SD) |
|---|---|
| water | 97.9 ± 1.0 |
| 1 mM EDTA solution, pH 7.5 | 95.2 ± 0.6 |
| 10 mM Tris buffer, pH 7.5 | 70.1 ± 2.5 |
| phosphate-buffered saline, pH 7.5 | 42.5 ± 3.8 |
| 300 mM lactose solution, pH 7.5 | 95.8 ± 0.4 |
| 1 mM EDTA/10 mM Tris buffer, pH 7.5 | 73.0 ± 0.8 |
| 1 mM EDTA/phosphate-buffered saline, pH 7.5 | 47.4 ± 2.6 |
| 300 mM lactose/1 mM EDTA solution, pH 7.5 | 95.6 ± 0.6 |

[a] DNA samples were directly lyophilized without the presence of plga or undergoing microsphere preparations.
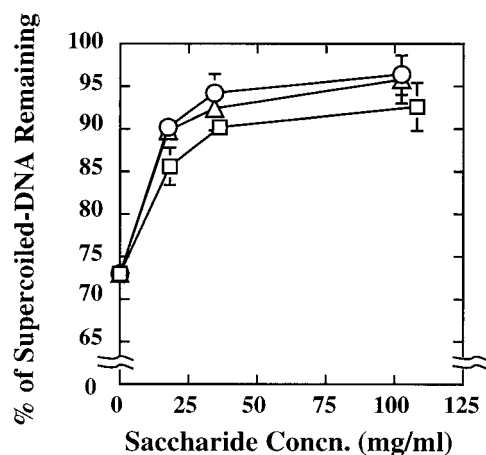


Figure 2—Effect of saccharides on DNA stability during lyophilization (DNA in TE buffer with increasing saccharide concentration). ○: Glucose, □: lactose, and △: sucrose.

to degrade DNA by its crystallization.) Saccharides are known cryoprotectants for proteins during lyophilization,[26,27] and we reasoned they may protect DNA in a similar fashion. Therefore, the effect of saccharides on DNA stability upon lyophilization from TE buffer was examined. Figure 2 indicates that the DNA degradation was decreased with an increase in the amount of saccharide. Other disaccharides such as maltose, trehalose, and cellobiose showed DNA stabilization similar to the effect of lactose (data not shown). The addition of saccharides in the inner aqueous phase of the primary emulsion improved the supercoiled-DNA content of the microspheres, as well (vide supra). Note that the concentrations of glucose are 100, 200, and 600 mM while those of sucrose and lactose are 50, 100, and 300 mM. This indicates that DNA stability depends on the total mass of saccharide and not the molar concentration of sugar.

Acidic pH is also known to damage DNA by depurination.[28] Freezing of sodium phosphate buffer, initially at pH 7.0, may result in pH as low as 4.0,[29] and this process could potentially contribute to the observed plasmid degradation. However, DNA which was incubated for up to 60 min at room temperature in PBS (Figure 3) or TE (10 mM Tris/1 mM EDTA; data not shown) showed no degradation at either pH 7.4 or pH 4.0. Thus, we believe any pH changes in the solution upon freezing have negligible effect on the DNA stability during microsphere preparation.

**Characteristics of Microspheres Optimized for High Supercoiled-DNA Content**—We have found that both (1) the addition of lactose and EDTA to the DNA solution and (2) the cryopreparation procedure were important to stabilize DNA against degradation during homogenization and lyophilization. We manufactured mi-
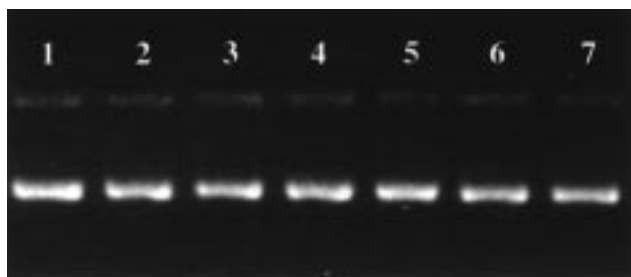
**Figure 3**—Agarose gel electrophoresis of DNA incubated at room temperature in PBS at pH 7.4 (lanes 1–4) and pH 4.0 (lanes 5–7) for 0 (lane 1), 10 (lanes 2, 5), 30 (lanes 3, 6), or 60 (lanes 4, 7) min.



**Figure 4**—Agarose gel electrophoresis of DNA in microspheres (MS) ($N = 3$). From left to right, lanes 1 and 11: 1 kb ladder; lanes 2–4: initial DNA; lanes 5–7: DNA in MS before lyophilization; lanes 8–10: DNA in MS after lyophilization.

**Table 4**—Effect of Excipients on Retaining Supercoiled DNA (SC DNA) under Microsphere Procedure Such as Homogenization and Lyophilization[a]

| | SC DNA remaining (initial% ± SD) | |
| --- | --- | --- |
| | before lyophilization | after lyophilization |
| water | 42.3 ± 0.7 | 39.0 ± 4.2 |
| 1 mM EDTA solution, pH 7.5 | 78.2 ± 1.7 | 68.3 ± 3.6 |
| phosphate-buffered saline, pH 7.5 | 49.1 ± 4.8 | 19.8 ± 0.4 |
| 300 mM lactose solution, pH 7.5 | 41.7 ± 2.4 | 20.8 ± 1.3 |
| 300 mM lactose/1 mM EDTA solution, pH 7.5 | 95.1 ± 0.7 | 88.6 ± 1.9 |

[a] Samples were prepared by cryopreparation, and the homogenization rate was 7000 rpm for 14 s ($n = 3$).

crospheres according to optimized conditions for the microsphere preparation described above to determine these combined effects. The characteristics of the microsphere such as particle size distribution, DNA conformation, and DNA encapsulation efficiency were determined.

Using the optimized conditions for microsphere preparation, the mean volume diameter of the microspheres was 4.8 $\mu$m, the remaining supercoiled DNA was 88% (Figure 4 and Table 4), and the DNA encapsulation efficiency was 89%. In comparison, using the standard double-emulsion preparation method (5000 rpm homogenization rate), the resulting microspheres had a mean volume diameter of 4.5 $\mu$m, a remaining supercoiled-DNA content of 39%, and a DNA encapsulation efficiency of 23%. It is apparent that cryopreparation prevents degradation of DNA and increases the encapsulation of DNA. These results suggest that the increase in DNA encapsulation efficiency is caused by preventing its diffusion out of the inner aqueous phase by freezing the primary emulsion. Furthermore, addition of DNA-nicking inhibitors to the DNA solution is important

to prevent DNA degradation during this microsphere manufacturing process. Ninety-five percent of supercoiled DNA was retained before lyophilization, and 88% of supercoiled DNA was retained after lyophilization as shown in Figure 4. In addition, Table 4 compares the supercoiled-DNA content in microspheres using water, EDTA, PBS, or lactose in the DNA solution. The results also show the reproducibility of the DNA stability data as compared to the isolated homogenization and lyophilization experiments (Tables 2 and 3).

In conclusion, a method for the microencapsulation of plasmid DNA is presented. The method, cryopreparation, maximizes both the retention of supercoiled DNA and the overall DNA encapsulation efficiency. Cryopreparation is characterized by the freezing of the aqueous phase of the primary emulsion. DNA-nicking inhibitors such as lactose and EDTA significantly improve the supercoiled-DNA content in the final sphere preparation.

## References and Notes

1. Friedmann, T. Progress Toward Human Gene Therapy. *Science* **1989**, *244*, 1275−1281.
2. Miller, A. D. Human Gene Therapy Comes of Age. *Nature* **1992**, *357*, 455−460.
3. Mulligan, R. C. The Basic Science of Gene Therapy. *Science* **1993**, *260*, 926−932.
4. Wilson, J. M. Vehicles for Gene Therapy. *Nature* **1993**, *365*, 691−692.
5. Crystal, R. G. The Gene as the Drug. *Nature Med.* **1995**, *1*, 15−17.
6. Langer, R. S. New Methods of Drug Delivery. *Science* **1990**, *249*, 1527−1533.
7. Kato, K.; Nakanishi, M.; Kaneda, Y.; Uchida, T.; Okada, Y. Expression of Hepatitis B Virus Surface Antigen in Adult Rat Liver. *J. Biol. Chem.* **1991**, *266*, 3361−3364.
8. Jong, Y. S.; Jacob, J. S.; Yip, K. P.; Gardner, G.; Seitelman, E.; Whitney, M.; Montgomery, S.; Mathiowitz, E. J. Controlled Release of Plasmid DNA. *J. Controlled Release* **1997**, *47*, 123−134.
9. Mathiowitz, E.; Jacob, J. S.; Jong, Y. S.; Carino, G. P.; Chickering, D. E.; Chaturvedi, P.; Santos, C. A.; Vijayaraghavan, K.; Montgomery, S.; Bassett, M.; Morrell, C. Biologically Erodable Microspheres as Potential Oral Drug Delivery Systems. *Nature* **1997**, *386*, 410−414.
10. Smith, T.; Zhang, Y.; Niven, R. Toward Development of a Non-Viral Gene Therapeutic. *Adv. Drug Del. Rev.* **1997**, *26*, 135−150.
11. Tabata, Y.; Ikada, Y. Phagocytosis of Polymer Microspheres by Macrophages. *Adv. Polym. Sci.* **1990**, *94*, 110−141.
12. Ciftci, K.; Kao, S.; Kumar, P.; Smiley, E.; Bonadio, J.; Levy, R. J. Polymer Based Gene Delivery Systems: An Effective Way to Introduce DNA into Mammalian Cells. *Pharm. Res.* **1997**, *14*, s639.
13. Hedley, M. L.; Curley, J.; Urban, R. Microspheres Containing Plasmid-Encoded Antigens Elicit Cytotoxic T-Cell Responses. *Nature Med.* **1998**, *4*, 365−368.
14. Xu, Y.; Szoka, F. C. Mechanism of DNA Release from Cationic Liposome/DNA Complexes Used in Cell Transfection. *Biochem.* **1996**, *35*, 5616−5623.
15. Yamaizumi, M.; Horwich, A. L.; Ruddle, F. H. Expression and Stabilization of Microinjected Plasmids Containing the Herpes Simplex Virus Thymidine Kinase Gene and Polyoma Virus DNA in Mouse Cell. *Mol. Cell Biol.* **1983**, *3*, 511−522.
16. Middaugh, C. R.; Evans, R. K.; Montgomery, D. L.; Casimiro, D. R. Analysis of Plasmid DNA from a Pharmaceutical Perspective. *J. Pharm. Sci.* **1998**, *87*, 130−146.
17. Sefton, M. V.; Brown, L. R.; Langer, R. S. Ethylene-Vinyl Acetate Copolymer Microspheres for Controlled Release of Macromolecules. *J. Pharm. Sci.* **1984**, *73*, 1859−1861.
18. Johnson, O. L.; Cleland, J. L.; Lee, H. J.; Charnis, M.; Duenas, E.; Jaworowicz, W.; Shepard, D.; Shahzamani, A.; Hones, A. J. S.; Putney, S. D. A Month-Long Effect from a Single Injection of Microencapsulated Human Growth Hormone. *Nature Med.* **1996**, *2*, 795−799.
19. Alonso, M. J.; Cohen, S.; Park, T. G.; Gupta, R. K.; Siber, G. R.; Langer, R. S. Determination of Release Rate of Tetanus Vaccine from Polyester Microspheres. *Pharm. Res.* **1993**, *10*, 945−953.

20. Schefer, U.; Ammann, D.; Pretsch, E.; Oesch, U.; Simon, W. Neutral Carrier Based Calcium-Selective Electrode with Detection limit in the Sub-Nanomolar Range. *Anal. Chem.* **1986**, *58*, 2282−2885.

21. Bell, C. F. *Principles and Applications of Metal Chelation*; Clarendon Press: Oxford, U.K., 1977; pp 75−91.

22. Price, P. A. Characterization of Ca and Mg Binding to Bovine Pancreatic Deoxyribonuclease A. *J. Biol. Chem.* **1972**, *247*, 2895−2899.

23. Brown, T. A. In *Gene Cloning*; T. A. Brown, Ed.; Chapman and Hall: New York, 1990; pp 30−46.

24. Hanes, J. Polymer Microspheres for Vaccine Delivery. Ph.D., Massachusetts Institute of Technology, 1996.

25. Murase, N.; Franks, F. Salt Precipitation During the Freeze-Concentration of Phosphate Buffer Solutions. *Biophys. Chem.* **1989**, *34*, 293−300.

26. Carpenter, J. F.; Prestrelski, S. J.; Anchordoguy, T. J.; Arakawa, T. Interactions of Stabilizers with Proteins During Freezing and Drying. *ACS Symp. Ser.* **1994**, *567*, 134−147.

27. Suzuki, T.; Imamura, K.; Yamamoto, K.; Satoh, T.; Okazaki, M. Thermal Stabilization of Freeze-Dried Enzymes by Sugars. *J. Chem. Eng. Jpn.* **1997**, *30*, 609−613.

28. Suzuki, T.; Ohsumi, S.; Makino, K. Mechanistic Studies on Depurination and Apurinic Site Chain Breakage in Oligodeoxyribonucleotides. *Nucleic Acids Res.* **1994**, *22*, 4997−5003.

29. Szkudlarek, B. A.; Anchordoquy, T. J.; Garcia, G. A.; Pikal, M. J.; Carpenter, J. F. pH Changes of Phosphate Buffer Solutions During Freezing and Their Influence on the Stability of a Model Protein, Lactate-Dehydrogenase. *Abstr. Pap. Am. Chem. Soc.* **1996**, *211*, 138-BIOT.

## Acknowledgments