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# Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles

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**Abstract**

Nanoparticles formulated from biodegradable polymers such as poly (lactic acid) and poly (D,L-lactide-co-glycolide) (PLGA) are being extensively investigated as non-viral gene delivery systems due to their sustained release characteristics and biocompatibility. PLGA nanoparticles for DNA delivery are mainly formulated using an emulsion-solvent evaporation technique. However, this formulation procedure results in the formation of particles with heterogeneous size distribution. The objective of the present study was to determine the relative transfectivity of the smaller- and the larger-sized fractions of nanoparticles in cell culture. PLGA nanoparticles containing a plasmid DNA encoding luciferase protein as a marker were formulated by a multiple emulsion-solvent evaporation method (mean particle diameter $= 97 \pm 3$ nm) and were fractionated using a membrane (pore size: 100 nm) filtration technique. The particles that passed through the membrane were designated as the smaller-sized nanoparticles (mean diameter $= 70 \pm 2$ nm) and the fraction that was retained on the membrane as the larger-sized nanoparticles (mean diameter $= 202 \pm 9$ nm). The smaller-sized nanoparticles showed a 27-fold higher transfection than the larger-sized nanoparticles in COS-7 cell line and a 4-fold higher transfection in HEK-293 cell line. The surface charge (zeta potential), cellular uptake, and the DNA release were almost similar for the two fractions of nanoparticles, suggesting that some other yet unknown factor(s) is responsible for the observed differences in the transfection levels. The results suggest that the particle size is an important factor, and that the smaller-sized fraction of the nanoparticle formulation predominantly contributes towards their transfection. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Non-viral vectors; Gene therapy; Biodegradable polymers; Cellular uptake

## 1. Introduction

Gene therapy, the introduction of an extraneous gene into a cell with the aim of replacing a lost cellular function or to introduce a new func-

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tionality, is fast becoming a reality (Rubanyi, 2001). However, achieving an efficient gene delivery into the target cell population or tissue without causing any vector-associated toxicity is critical to the success of gene therapy (Clark and Johnson, 2001). To achieve the above objective, various viral and non-viral vectors have been investigated (Kataoka and Harashima, 2001). Although viral vectors such as adenovirus, influenza virus and adeno-associated virus (Kochanek et al., 2001) are relatively more efficient in gene transfection than non-viral methods (Li and Huang, 2000), their toxicity and immunogenicity are the major concerns. Therefore, non-viral vectors such as liposomes, cationic block copolymers, polymer complexes, and micro- and nanoparticles have gained importance because they are relatively safe and are easy to formulate (Luo et al., 1999; Maheshwari et al., 2000). More recently, biodegradable nanoparticles formulated using different polymers such as chitosan (Mao et al., 2001; Roy et al., 1999), gelatin (Truong-Le et al., 1998), and other biodegradable polymers (Roy et al., 1999; Cohen et al., 2000) are being investigated as non-viral gene delivery systems because of the possibility of achieving safe and sustained gene transfection.

We have been investigating biodegradable nanoparticles formulated from poly (D,L-lactide-co-glycolide) (PLGA), an FDA approved biocompatible and biodegradable polymer, as a non-viral gene delivery system (Labhasetwar et al., 1999). Nanoparticles, because of their subcellular size, are effectively endocytosed by the cells which could result in higher cellular uptake of the entrapped DNA (Panyam et al., 2002b; Davda and Labhasetwar, 2002). Recently, we have demonstrated the rapid escape of PLGA nanoparticles from the endo-lysosomal compartment into cytoplasm, suggesting the suitability of nanoparticles as a gene delivery vector (Panyam et al., 2002b). Since the DNA is encapsulated inside the polymeric matrix, it would be protected from extracellular and intracellular nuclease degradation (Hedley et al., 1998). The DNA entrapped in nanoparticles is released slowly with the hydrolysis of the polymer matrix due to the cleavage of the ester bonds. It is hypothesized that the slow release of DNA from nanoparticles intracellularly would be effective in achieving sustained gene expression in the target tissue. Sustained and regulated gene expression is probably more important in treating certain localized disease conditions such as the cardiac and limb ischemia by inducing neovascularization in the damaged tissue using genes encoding pro-angiogenic growth factors (Richardson et al., 2001). Similarly, sustained gene expression has been shown to be effective in bone regeneration, which could be useful to repair fractured bones (Bonadio et al., 1999). Restenosis, a vasculoproliferative condition that occurs following coronary balloon angioplasty procedure, is another example of a pathological condition where sustained gene expression in the target artery could be more effective (Ohno et al., 1994; Klugherz et al., 2000).

Various formulation factors and characteristics of nanoparticles could influence the transfectivity of nanoparticles. One of the important parameters that could affect the transfectivity of nanoparticles is their size. The particle size has been an important consideration while formulating other particulate type systems such as DNA-polymer (Dauty et al., 2001) and lipid complexes (Lee et al., 2001) and liposomes (Sakurai et al., 2000). Our studies and that of others have shown that the particle size significantly affects their cellular and tissue uptake (Desai et al., 1997; Zauner et al., 2001), and in some cell lines, only the submicron size particles are taken up efficiently but not the larger size microparticles (e.g. Hepa 1-6, HepG2, and KLN 205) (Zauner et al., 2001). Nanoparticles prepared by emulsion-solvent evaporation technique using PLGA polymer usually results in the formation of nanoparticles with heterogeneous particle size distribution. We hypothesized that the transfectivity of the different sized nanoparticle fractions in the formulation could be different. Therefore, the objective of the present study was to determine the relative transfectivity of the smaller- and the larger-sized fractions of nanoparticles in cell culture.

## 2. Materials and methods

### 2.1. Materials

PLGA (MW 143900 Da, copolymer ratio 50:50) was purchased from Birmingham Polymers, Inc. (Birmingham, AL). Acetylated bovine serum albumin (Ac-BSA), MEM non-essential amino acid solution (100 ×), and polyvinyl alcohol (PVA, average MW 30000–70000) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS, heat inactivated), 1 × trypsin-EDTA, Dulbecco's modified essential medium (DMEM), and penicillin-streptomycin were obtained from Gibco-BRL (Grand Island, NY). African green monkey kidney epithelial (COS-7) and human embryonic kidney epithelial (HEK-293) cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Luciferase plasmid with simian virus 40 (SV40) promoter (pGL3), DNA molecular weight markers, cell culture lysis reagent (CCLR, 5 ×), luciferase assay kit, and the recombinant luciferase protein were purchased from Promega (Madison, WI). 6-Coumarin was purchased from Polyscience Inc. (Warrington, PA).

### 2.2. Methods

#### 2.2.1. Formulation of nanoparticles containing plasmid DNA

Plasmid pGL3 containing firefly luciferase gene under the control of SV40 promoter was used as a marker DNA to study the transfectivity of nanoparticles. The DNA-loaded nanoparticles were formulated by a double emulsion-solvent evaporation technique (Labhasetwar et al., 1999). In brief, an aqueous solution containing 1 mg of DNA and 2 mg of Ac-BSA in 200 μl of Tris–EDTA (TE) buffer (pH 8.0) was emulsified into 1 ml of polymer solution in chloroform (30 mg PLGA/ml) using a probe sonicator (XL 2015 sonicator® ultrasonic processor, Misonix Inc., Farmingdale, NY) at 55 W of energy output for 1 min over an ice bath. The primary emulsion was then emulsified into 6 ml of 2% w/v aqueous solution of PVA using sonication as above for 5 min to form a multiple (water-in-oil-in-water) emulsion. The emulsion was stirred at room temperature for ~18 h to evaporate chloroform. Nanoparticles thus formed were recovered by ultracentrifugation at 35000 rpm (Beckman Optima™ LE-80K, Beckman Instruments, Inc., Palo Alto, CA), washed three times with distilled water to remove PVA and unentrapped DNA, resuspended in water, and lyophilized for 48 h (VirTis Co., Inc. Freeze Dryer, Gardiner, NY). All the washings following the recovery of nanoparticles were saved to determine the total DNA that was not encapsulated into nanoparticles (see Section 2.2.5 for DNA loading).

#### 2.2.2. Fractionation of nanoparticles into different sizes

Lyophilized nanoparticles were resuspended in 10 ml of water by sonication for 2 min as above. Nanoparticles were then fractionated into two sizes by passing the suspension through a Durapore® VVPP membrane of porosity cut off 100 nm (Millipore, Bedford, MA) using an Amicon® membrane filtration system (Millipore). Fractionation of nanoparticles was carried out at a nitrogen pressure of 50 psi and the flow rate of 0.06 ml/min. When the suspension volume in the Amicon® container reached ~1 ml, additional 10 ml water was added into the suspension and the fractionation was continued until the volume in the container reached ~1 ml. The nanoparticle fraction that passed through the membrane and that retained on the membrane were collected separately and were lyophilized as above. For the cellular uptake studies, a formulation of nanoparticles that contained a fluorescent marker in addition to DNA was used. The dye (6-coumarin, 50 μg) was dissolved in the PLGA polymer solution prior to emulsification. Thus, the nanoparticle formulation used for the transfection studies and for the cellular uptake studies were essentially similar in composition except that the latter formulation contained the fluorescent dye. The dye serves as a sensitive marker for nanoparticles and was used in our previous studies to quantitatively determine the cellular and tissue uptake of nanoparticles (Panyam et al., 2002a; Davda and Labhasetwar, 2002).

*2.2.3. Determination of particle size and surface charge of nanoparticles*

The particle size of the unfractionated and the fractionated nanoparticles was determined by both dynamic light scattering technique and transmission electron microscopy (TEM). For TEM, a drop of nanoparticle suspension was placed on to the TEM grid and the particles were visualized following negative staining with 2% uranyl acetate (Electron Microscopy services, Ft. Washington, PA) using a Philips 201® TEM (Philips/FEI Inc., Briarcliff Manor, NY). Diameter of 100–150 nanoparticles from five to seven different TEM fields was measured to determine the mean particle size of nanoparticles. To measure nanoparticle size using dynamic laser light scattering, a nanoparticle suspension (0.5 mg/ml in distilled water) was subjected to particle size analysis using a Zeta Plus™ particle size analyzer (Brookhaven Instruments Corp., Holtsville, NY). Zeta potential (surface charge) of nanoparticles (0.5 mg/ml nanoparticles in water) was determined using a Zeta Plus™ zeta potential analyzer.

*2.2.4. Determination of nanoparticle bound PVA*

The amount of PVA associated with nanoparticles was determined using a colorimetric method described by Joshi et al. (1979). About 2 mg of lyophilized nanoparticle sample was digested in 2 ml of 0.5N NaOH for 15 min at 60 °C. The solution was neutralized with 900 μl of 1N HCl and then diluted to 5 ml with distilled water. To the above solution, 3 ml of 0.65 M boric acid, 0.5 ml of $I_2$/KI (0.05/0.15 M) and 1.5 ml of distilled water were added. Following incubation for 15 min at room temperature, the absorbance of the solution was measured at 690 nm using an UV spectrophotometer. The standard plot was prepared using PVA solution at different concentrations (0–30 μg/ml) that were treated similarly. The amount of PVA associated with nanoparticles was then expressed as % w/w.

*2.2.5. Determination of DNA loading, encapsulation efficiency, and DNA conformation following encapsulation*

The DNA loading in nanoparticles was determined from the total amount of DNA added in the formulation and the DNA amount that was not encapsulated. For this, the concentration of DNA in the washings was determined by measuring the UV absorbance at 260 nm with the washings from the control nanoparticles formulated without DNA as a blank. Direct extraction of DNA from nanoparticles was also tried by two different methods. A sample of nanoparticles was digested in 0.5N NaOH at 60 °C and the aliquots were taken at 15 min, 30 min, 1 h, 2 h, and 24 h. The aliquots were centrifuged (10 min at 4 °C and 14000 rpm, Eppendorf® 5417R, Brinkmann Instruments, Westbury, NY) and the supernatant from each tube was assayed for the DNA levels at 260 nm using an UV spectrophotometer. The digest from control nanoparticles treated in the same manner served as a blank. In another method, a sample of nanoparticles was dissolved in chloroform and the chloroform layer was extracted repeatedly (five times) with TE buffer (pH 8.0). The DNA levels in the extract were determined as above. The DNA extracted using the above method was used to determine the conformation of the encapsulated DNA by agarose gel electrophoresis. The conformation of the DNA, which did not get encapsulated in nanoparticles and leached out in the aqueous PVA solution during their formulation, was also determined. A 20 μl aliquot of the extracted DNA and the DNA from the nanoparticle washing were loaded on to a 0.8% agarose gel in electroporation buffer with stock plasmid DNA solution as a control. The gel was visualized under UV following staining with ethidium bromide solution and photographed.

*2.2.6. In vitro release of DNA from nanoparticles*

In vitro release of the DNA from the larger and smaller-sized fractions of nanoparticles was studied by incubating 0.15 mg of the respective fractions with 0.5 ml of TE buffer in Eppendorf® tubes at 37 °C in an Environ Orbital Shaker (Lab Line, Melrose Park, IL) set at 100 rpm. Separate tubes were used for each data point. At predetermined time intervals, the nanoparticle suspension was centrifuged and the amount of DNA released in the supernatant was analyzed by PicoGreen® assay (Promega).

*2.2.7. Transfection studies and determination of luciferase protein levels*

COS-7 and HEK-293 cells were grown in DMEM supplemented with 10% FBS, 100 μg/ml penicillin G, 100 μg/ml streptomycin, and 5% MEM non-essential amino acids. For transfection studies, cells were cultured in 24-well plate 1 day prior to transfection. The transfection was carried out at ~70% confluency. A nanoparticle suspension was prepared in the serum free medium (3 mg in 500 μl) using a water bath sonication for 10 min (FS140, Fisher Scientific, Pittsburgh, PA). The nanoparticle suspension was then diluted to 12 ml with complete DMEM medium. The medium in the wells was replaced with 1 ml of nanoparticle suspension (equivalent to about 6.8 μg DNA/well). The dose of nanoparticles used for the transfection was based on the preliminary dose-response study. The medium was changed on every 3rd day for HEK-293 cell line with no further addition of nanoparticles. To measure the luciferase protein levels, the cells were washed twice using 1× phosphate buffer saline (PBS) and lysed using 1× CCLR (Promega). To each 20 μl of the cell lysate sample, 100 μl of the reconstituted luciferase assay substrate (Promega) was added and the chemiluminiscence intensity was measured immediately using a luminometer (TD 20/20, Promega). The amount of luciferase protein was determined from the standard plot prepared using a recombinant luciferase protein. The total cell protein was determined using a BioRad® protein assay kit (BioRad, Hercules, CA) and the data were represented as luciferase protein levels in fg per mg cell protein.

*2.2.8. Cellular nanoparticle uptake studies*

For the cellular uptake study, a formulation of DNA loaded nanoparticles containing 6-coumarin as a fluorescent marker was used. COS-7 cells were incubated with a suspension of nanoparticles at the same dose used for the transfection study for 1 h, washed twice with 1× PBS, and then lysed using 100 μl/well of 1× CCLR. One hour was selected for the incubation of nanoparticles because the previous studies have shown almost a saturation uptake of nanoparticles during this time period (Davda and Labhasetwar, 2002). Intracellular nanoparticle uptake was determined as described previously (Davda and Labhasetwar, 2002). In brief, a 5 μl aliquot of each sample was used to determine the total cell protein using BioRad® assay and the remaining portion was lyophilized for 24 h. The dye (6-coumarin) from the nanoparticles in the cell lysates was extracted by incubating each cell lysate sample with 1 ml of methanol at 37 °C for 24 h at 100 rpm in an Environ® lab shaker (Labline, Melrose Park, IL). The samples were centrifuged (14000 rpm for 10 min at 4 °C in an Eppendorf® microcentrifuge) to remove the cell debris and the supernatant from each sample was analyzed for the 6-coumarin levels using a high performance liquid chromatography (HPLC) as described in our previous studies (Davda and Labhasetwar, 2002). The uptake was represented as nanoparticle amount in μg normalized to per mg of total cell protein.

*2.2.9. Statistical methods*

Student *t*-test was used to test the significance of difference in the transfection efficiency and uptake of the smaller and the larger-sized particles. A *P*-value less than 0.05 was accepted as statistically significant. All data analyses were done using MINITAB® statistical software (Minitab Inc., State College, PA).

## 3. Results and discussion

*3.1. Characterization of nanoparticles containing plasmid DNA*

The unfractionated nanoparticles, when analyzed by dynamic light scattering, demonstrated a bimodal size distribution with particles in two size ranges: 95–130 nm and 330–450 nm (Fig. 1A). Following fractionation, both the fractions demonstrated unimodal particle size distribution. The fraction that passed through the membrane was designated as the smaller-sized nanoparticles and the fraction that was retained on the membrane was designated as the larger-sized nanoparticles. The smaller-sized fraction had a mean hydrodynamic diameter of 148.7 nm (polydispersity = 0.115) (Fig. 1B) while the larger-sized frac-

tion had a mean hydrodynamic diameter of 298.2 nm (polydispersity = 0.224) (Fig. 1C). Although the smaller-sized nanoparticles were passed through the membrane of 100 nm porosity, the particle size measurement showed a mean diameter larger than 100 nm by the above method. The discrepancy in the size of nanoparticles is because the dynamic light scattering method gives the hydrodynamic diameter rather than the actual diameter of nanoparticles. The particle size discrepancy is further evident from the TEM of the unfractionated and the fractionated nanoparticles.

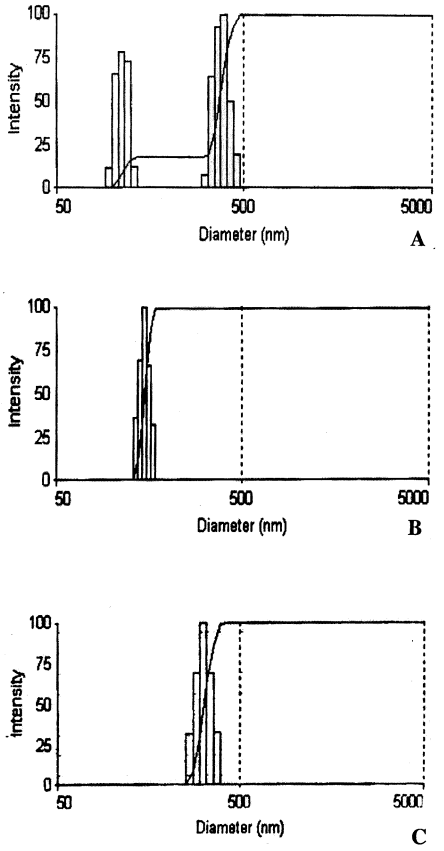


Fig. 1. Particle size distribution of (A) unfractionated nanoparticles, (B) nanoparticles passing through the membrane, and (C) nanoparticles retained on the membrane. Particle size distribution was measured by dynamic light scattering. The unfractionated nanoparticles have bimodal particle size distribution whereas fractionated nanoparticles have unimodal size distribution. The horizontal lines across the graphs represent the cumulative fraction of the total intensity of the different sized nanoparticles

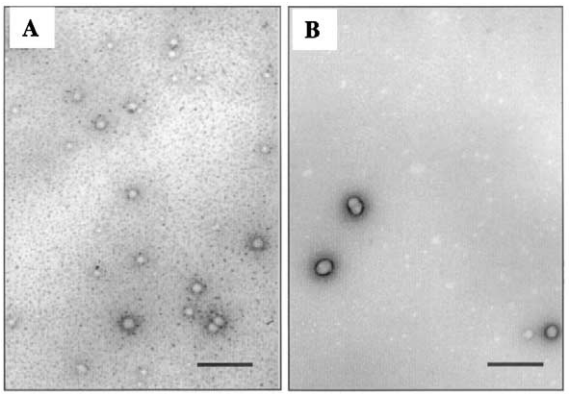


Fig. 2. Transmission electron microscopy of fractionated smaller-sized (A) and larger-sized (B) nanoparticles. Bar represents 500 nm. Magnification 14000 ×.

The mean diameter of the unfractionated nanoparticles was $97 \pm 3$ nm whereas that of the lower-sized fraction was $70 \pm 2$ nm (Fig. 2A) and the larger-sized fraction was $202 \pm 9$ nm (Fig. 2B). Thus the mean nanoparticle diameter measured using TEM is significantly smaller than the mean diameter obtained with the dynamic light scattering method.

The PVA associated with nanoparticles could contribute towards the hydrodynamic diameter of nanoparticles. In this study, it was found that $9.1 \pm 0.9\%$ w/w (mean $\pm$ SEM, $n = 6$) PVA remains associated with nanoparticles and is not washable. The PVA is known to form layers of aggregates ($\sim 5$ layers) around the surface of nanoparticles contributing towards the hydrodynamic diameter of nanoparticles (Zambaux et al., 1998). Recently, we have shown that the amount of PVA bound to nanoparticles affect their physical (e.g. zeta potential, hydrophilicity) as well as cellular uptake properties. Nanoparticles with greater amount of bound PVA have been found to have reduced cellular uptake (Sahoo et al., 2002). Harvie et al. (2000) have shown that the transfection efficiency of lipid-DNA complex is reduced following conjugation of the lipid to poly (ethylene glycol). The reduced cellular uptake of the conjugated lipid–DNA complex due to steric hindrance has been attributed to the reduced transfection efficiency. In our studies, the reduced cellular uptake of nanoparticles with higher PVA

bound nanoparticles has been attributed to the increase in hydrophilicity of nanoparticles with the increase in the amount of nanoparticle bound PVA. Therefore, it would be interesting to determine in the future studies the effect of nanoparticle-bound PVA on the transfection efficiency of nanoparticles.

Zeta potentials of the DNA-loaded unfractionated ($-23 \pm 1$ mV, mean of five readings $\pm$ SEM), the smaller-sized ($-20.8 \pm 1.6$ mV) and the larger-sized ($-20.6 \pm 1.5$ mV) nanoparticle fractions were almost similar, suggesting that there was no difference in the surface charge characteristics of these nanoparticles. The DNA loading in the nanoparticles, as determined by the indirect method of analysis, was 2.1–2.7% w/w with an entrapment efficiency of 78–83%. The loading represents the amount of DNA present per unit weight of nanoparticles whereas the efficiency of entrapment represents the percent of the total added DNA that has been entrapped into nanoparticles. Attempts to determine the DNA loading in nanoparticles by direct methods were not successful either because the DNA degraded under the conditions used for digesting the nanoparticles or due to the precipitation of PLGA–PVA complex along with DNA at the organic solvent–TE buffer interface resulting in the incomplete extraction of DNA. The above problem of extraction of macromolecules especially from nanoparticles has been reported by other investigators (Cohen et al., 2000), and hence the indirect method of DNA loading is commonly used to overcome the problem (Perez et al., 2001; Klugherz et al., 2000).

The DNA that leached out in the washings and that extracted from nanoparticles showed bands corresponding to that of the supercoiled and circular forms of DNA (Fig. 3A and B). The results thus suggest that sonication did not cause the fragmentation of DNA during the formulation of nanoparticles, and the DNA was probably protected due to its encapsulation within the polymer in the emulsion. Sonication of DNA in solution under similar conditions resulted in a complete fragmentation of DNA. Protection of the DNA structure during emulsification is important as fragmentation of DNA could affect the transfectivity of nanoparticles (Walter et al., 1999). While there was some open circular form of the DNA present in the stock, agarose gel electrophoresis results show that the encapsulation results in a partial transformation of DNA from the supercoiled to the open circular form. This partial transformation of DNA following encapsulation has been reported for PLGA microspheres and has been attributed to the encapsulation procedure (homogenization) and lyophilization (Ando

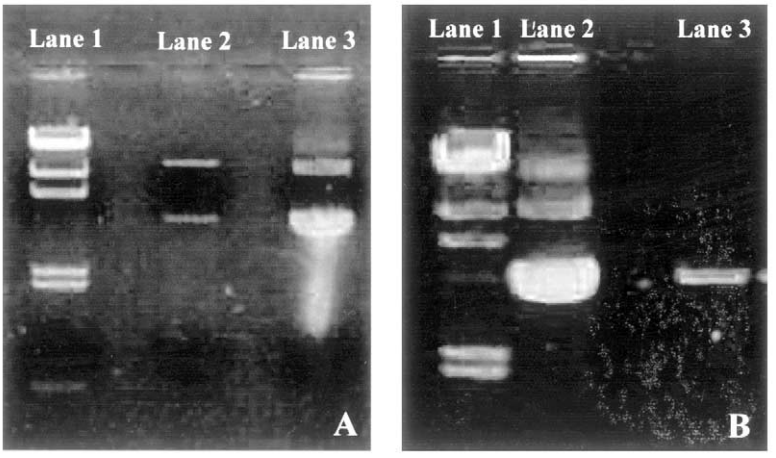


Fig. 3. Agarose gel electrophoresis of DNA extracted from nanoparticles (A) (lane 1: molecular weight markers, lane 2: extracted DNA, lane 3: stock plasmid DNA) and from nanoparticle washings (B) (lane 1: molecular weight markers, lane 2: stock plasmid DNA, lane 3: nanoparticle washings).

et al., 1999). Similar transformation of the DNA following encapsulation has been reported by other investigators also (Cohen et al., 2000; Cherng et al., 1999; Bergan et al., 2000). However, this partial transformation of DNA is not expected to affect the transfectivity of nanoparticles as Cohen et al. (2000) have demonstrated that the difference in the transfection levels of the supercoiled and relaxed forms of the DNA extracted from nanoparticles is statistically insignificant. Also, in another study, it was demonstrated that DNA in supercoiled or relaxed form formulated with cationic lipids do not show any significant difference in the transfection efficiency in cell culture (Bergan et al., 2000).

### 3.2. Effect of particle size on transfection, DNA release, and cellular uptake

The transfection studies in COS-7 showed a 27-fold higher luciferase protein levels for the fractionated smaller-sized nanoparticles as compared to the larger-sized nanoparticles ($P < 0.05$, $n = 6$) or the unfractionated particles (Fig. 4A) for the same dose of nanoparticles. The fact that the fraction of the smaller-sized particles is significantly lower in the unfractionated nanoparticle preparation (less than 5% by weight) may explain the similar transfection levels observed for the unfractionated and the larger-sized nanoparticles (Fig. 4A). In the transfection studies, however, equal weight of the smaller-sized and the larger-sized nanoparticles was used and hence the difference in the transfectivity of the two sized nanoparticles could be seen. The transfection studies in COS-7 cells could not be continued beyond 2 days because the cells reached confluency and began to detach.

In HEK-293 cell line, the smaller-sized nanoparticles showed a 4-fold higher transfection as compared to the larger-sized nanoparticles ($P < 0.05$, $n = 6$) (Fig. 4B). The transfection level increased slowly and reached a peak in 5–7 days post-transfection. The cells started detaching thereafter and hence the transfection studies could not be continued beyond 1 week. The sustained gene expression in HEK-293 cells seems to be due to the slow release of DNA from the nanoparticles. Similar studies using FuGene™6 (Roche Diagnostic, Indianapolis, IN), a commercially available transfecting agent, showed peak luciferase level at 2 days, which then rapidly declined

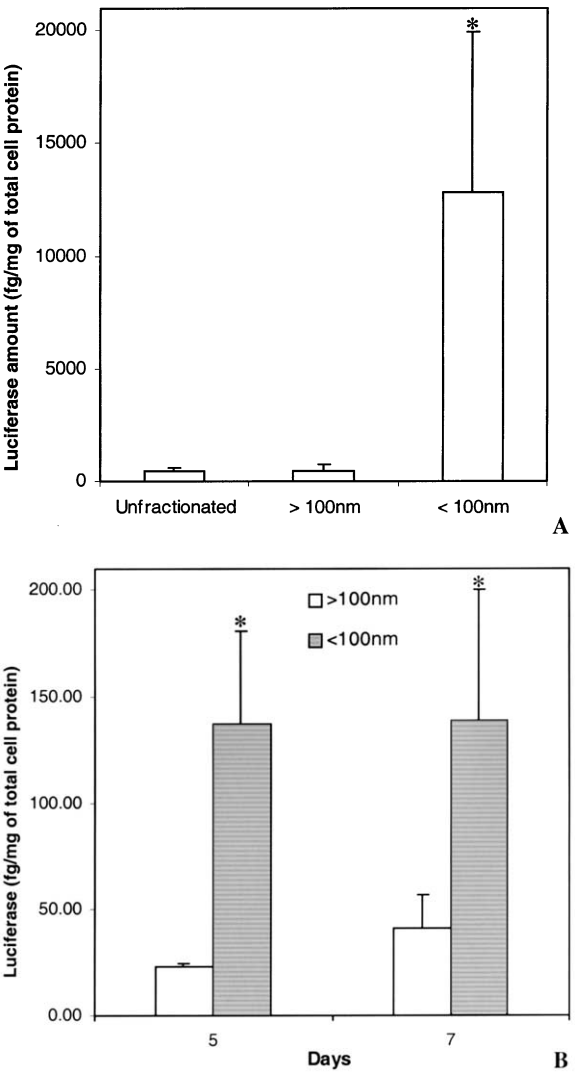


Fig. 4. (A) Transfection with unfractionated and fractionated smaller- and larger-sized nanoparticles in COS-7 cell line. The luciferase protein levels were measured two days after the transfection. Results are expressed as mean ± SEM ($n = 6$, * $P < 0.05$ compared to larger-sized and unfractionated nanoparticles). (B) Transfection with fractionated smaller- and larger-sized nanoparticles in HEK-293 cell line. The luciferase protein levels were measured at 5 and 7 days post-transfection. Results are expressed as mean ± SEM ($n = 6$, * $P < 0.05$ compared to larger-sized nanoparticles).

(data not shown), supporting our hypothesis of sustained gene expression using nanoparticles.

Further, about 165-fold difference ($78 \pm 30.5$ fg/mg in HEK-293 vs. $12805 \pm 7128$ fg/mg in COS-7 for smaller-sized nanoparticles) in the transfection levels observed in the two cell lines, for the same dose of nanoparticles and for the same duration (2 days) of transfection, suggests that the nanoparticle-mediated gene transfection is cell line-dependent. The cell line-dependent variation in transfection levels has also been reported for other gene delivery systems. It is possible that COS-7 is a relatively rapidly dividing cell line compared to HEK-293, and hence there is greater nuclear uptake of DNA released from nanoparticles in COS-7 than in HEK-293 during cell division. The above possibility has been suggested for cationic lipid-mediated gene transfer (Escriou et al., 2001).

In order to determine the mechanism of higher transfectivity of the smaller-sized nanoparticles compared to that of the larger-sized nanoparticles, we determined the DNA release from the two fractions of nanoparticles and their cellular uptake in COS-7 cells. The DNA release study shows that the larger-sized nanoparticles have slightly higher extent of DNA release than from an equal weight of the smaller-sized nanoparticles (Fig. 5). In this study, the release study was carried out for 7 days because the transfection study was carried out for a maximum period of 7 days. However, in our previous study, a sustained release of DNA from PLGA nanoparticles was demonstrated with a 26% cumulative release occurring in the 1st hour as a burst phase, followed by a gradual elution with a 82% cumulative release in 17 days (Labhasetwar et al., 1999). Similar sustained DNA release pattern for PLGA nano- and microparticles has been reported by other investigators (Wang et al., 1999; Cohen et al., 2000). Based on the in vitro release study, the available DNA for transfection is not significantly different for the two fractions of nanoparticles and does not seem to be the factor responsible for higher transfection observed with the smaller-sized nanoparticles. The cellular uptake study demonstrated that the uptake of the smaller- and the larger-sized nanoparticles were almost similar ($33.2 \pm 6.5$ μg nanoparticles/mg vs. $27.5 \pm 4.7$ μg nanoparticles/mg of total cell protein, respectively, mean $\pm$ SEM, $n = 6$, $P > 0.05$).

Since the surface charge (zeta potential), cellular uptake, and the DNA release were almost similar for the two fractions of nanoparticles, these factors do not seem to be responsible for the observed differences in the transfection levels. One of the possible reasons for the observed higher transfection efficiency for the smaller-sized fraction could be due to the difference in the total number of particles present in the cells for the same mass of nanoparticles taken up. Based on the mean particle diameter of the two fractions of nanoparticles and the above cellular uptake data, it could be estimated that the number of the smaller-sized nanoparticles taken up by the cells would be about 20-fold greater than the larger-sized nanoparticles ($350 \times 10^8$ vs. $17 \times 10^8$ per mg cell protein for smaller- and larger-sized nanoparticles, respectively) (Müller, 1991).

Various investigators have reported the importance of size of plasmid DNA and DNA carriers for gene transfection (Kreiss et al., 1999; Cherng et al., 1999). Plasmid DNA of smaller size was previously shown to have higher transfection efficiency. It was suggested that the greater extent of

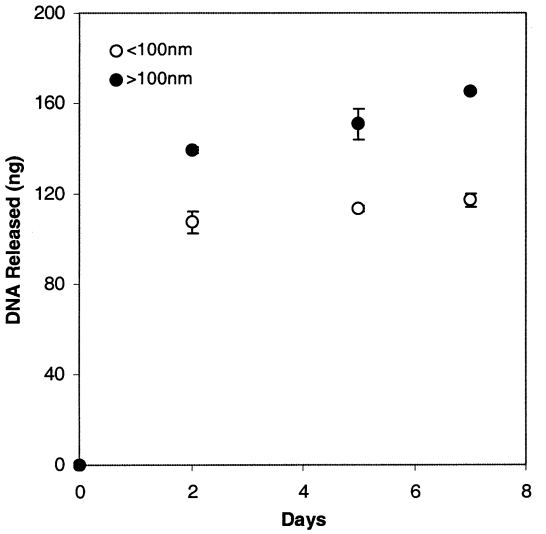


Fig. 5. In vitro release of DNA from smaller-sized and larger-sized nanoparticles. Data as mean $\pm$ SEM ($n = 3$).

the DNA release from cationic lipids as compared to that from larger size DNA complexes, or greater intracellular migration of smaller size DNA from cytoplasm to nucleus or both of the above mechanisms could be responsible for the observed higher transfection of the smaller size DNA (Kreiss et al., 1999). Cherng et al. have reported that the optimal transfection efficiency of the poly ((2-dimethylamino) ethyl methacrylate) (PDMAEMA)-plasmid particles was at PDMAEMA-plasmid ratio of 3 (w/w) containing slightly positively charged particles with a narrow size distribution and average diameter of 150 nm. However, PDMAEMA-plasmid ratios less than 1.5 (w/w) resulted in rather large particles with high polydispersity index. In their study, it has been suggested that the larger particles are too voluminous to be taken up by the cell via endocytosis resulting in lower transfection. Future studies aimed at investigating the intracellular distribution of the two fractions of nanoparticles could reveal the reason for the observed difference in the transfection efficiency of the two different nanoparticle fractions.

## 4. Conclusions

In this study, we have shown that the double-emulsion solvent evaporation technique commonly used for PLGA nanoparticle formulation results in a heterogeneous particle-size distribution and that the smaller-sized fraction of nanoparticles ($< 100$ nm) has significantly higher transfection efficiency as compared to the larger-sized fraction of nanoparticles ($> 100$ nm). The greater transfection of the smaller-sized fraction as compared to the larger-sized fraction of nanoparticles does not seem to be related to their surface properties (zeta potential), cellular uptake or the rate and extent of release of DNA. Although the method used in this study to fractionate nanoparticles may not be commercially practical since it provides low yield (less than 5% on weight basis) of the smaller-sized nanoparticles, the results of the study are important because they signify the importance of particle size in nanoparticle-mediated gene transfection. The results suggest that formulating nanoparticles of smaller diameter is critical to improving the efficiency of nanoparticle-mediated transfection.

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