Critical Determinants in PLGA/PLA Nanoparticle-Mediated Gene Expression

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Received June 30, 2003; accepted October 7, 2003

Purpose. The aim of the study was to determine the critical determinants in nanoparticle-mediated gene transfection. It was hypothesized that different formulation parameters could affect the nanoparticle characteristics and hence its gene transfection.

Methods. Nanoparticles encapsulating plasmid DNA encoding for firefly luciferase were formulated using polylactide (PLA) and poly (D,L-lactide-*co*-glycolide) (PLGA) polymers of different compositions and molecular weights. A multiple-emulsion solvent-evaporation method with polyvinyl alcohol (PVA) as an emulsifier was used to formulate DNA-loaded nanoparticles. Gene expression of nanoparticles was determined in breast cancer (MCF-7) and prostate cancer (PC-3) cell lines.

Results. Nanoparticles formulated using PLGA polymer demonstrated greater gene transfection than those formulated using PLA polymer, and this was attributed to the higher DNA release from PLGA nanoparticles. Higher-molecular-weight PLGA resulted in the formation of nanoparticles with higher DNA loading, which demonstrated higher gene expression than those formulated with lower-molecular-weight PLGA. In addition, the nanoparticles with lower amount of surface-associated PVA demonstrated higher gene transfection in both the cell lines. Higher gene transfection with these nanoparticles was attributed to their higher intracellular uptake and cytoplasmic levels. Further study demonstrated that the molecular weight and the degree of hydrolyzation of PVA used as an emulsifier also affect the gene expression of nanoparticles.

Conclusions. Results thus demonstrate that the DNA loading in nanoparticles and its release, and the surface-associated PVA influencing the intracellular uptake and endolysosomal escape of nanoparticles, are some of the critical determinants in nanoparticle-mediated gene transfection.

KEY WORDS: nonviral gene delivery; biodegradable and biocompatible polymers; sustained release; cancer therapy

INTRODUCTION

Toxicity and immunogenicity concerns associated with viral vectors have led to an active interest in nonviral vectors for gene delivery (1–3). Among the many nonviral systems currently being investigated, biodegradable polymeric nanoparticles with entrapped plasmid DNA have shown the potential for achieving sustained gene expression (4–6). Nanoparticles are colloidal particles in the nanometer size range and contain a plasmid DNA of interest entrapped in their polymer matrix (5). Although matrix-type nanoparticles have been formulated using different polymers (7), nanoparticles formulated from poly(D,L-lactide-*co*-glycolide) (PLGA) and polylactide (PLA) are especially of interest for gene delivery because of their biocompatibility, biodegradability, and sustained-release characteristics.

We have previously studied the nanoparticle-mediated gene transfection both *in vitro* (6) and *in vivo* (8) and have also determined the influence of particle size of nanoparticles on gene transfection *in vitro* (5). Other groups have also investigated the transfection efficiency of gene-loaded PLGA nanoparticles in comparison to the naked DNA and liposomal formulations both *in vitro* and *in vivo* (4). However, the influence of various formulation parameters on gene transfection, which could be critical to enhancing the efficiency of nanoparticle-mediated gene transfection, has not been thoroughly examined before.

We have recently demonstrated that following their uptake into the cells through an energy-dependent endocytic process, PLGA-nanoparticles rapidly escape the endolysosomes into the cytoplasm (6). Nanoparticles are anionic at physiologic pH; however, in the acidic pH of endolysosomes, nanoparticles acquire a net positive charge. This cationization of nanoparticle surface selectively in the endolysosomal compartment is hypothesized to result in the localized destabilization of the endosomal membrane leading to escape of nanoparticles. In the transmission electron microscopy of the cell, nanoparticles were seen to interact with the endosomal vesicles only in the secondary endosomes, where pH is acidic (pH ~4), but not in the primary endosomes where the pH is 7.4. Furthermore, it was demonstrated that nanoparticles that do not show charge reversal with pH (e.g., polystyrene nanoparticles) were not seen to escape into the cytoplasmic compartment, thus substantiating our proposed hypothesis. The uptake of nanoparticles was inhibited in the presence of metabolic inhibitors (sodium azide and deoxyglucose), confirming the endocytic process of uptake of nanoparticles. The nanoparticle uptake was determined to be partly through fluid-phase pinocytosis and partly through clathrin-coated pits in vascular smooth muscle cells (6). Nanoparticles that escaped the endosomes into the cytoplasmic compartment are hypothesized to slowly release the entrapped DNA, resulting in sustained gene expression.

Recently, we have demonstrated that polyvinyl acetate (PVA), which is a commonly used emulsifier in the formulation of nanoparticles, remains associated with the nanoparticle surface. This occurs because the hydrophobic portion of PVA anchors into the nanoparticle matrix during their formulation, could not be washed away, and therefore forms the nanoparticle interface. Other investigators, with similar formulation of nanoparticles, have demonstrated the presence of PVA at the nanoparticle surface using x-ray photoelectron spectroscopy (9). PVA has been estimated to form multilayers around the nanoparticle surface (10). Our studies have demonstrated that the residual surface-associated PVA affects the physical properties of nanoparticles as well as their cellular uptake (11). Therefore, it was hypothesized that the surface-associated PVA and its concentration, type (degree of hydrolyzation), and molecular weight could influence the gene transfection of nanoparticles.

Thus, the factors influencing the intracellular uptake of nanoparticles and their distribution, and DNA loading in

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0724-8741/04/0200-0354/0 © 2004 Plenum Publishing Corporation

nanoparticles and its release, could affect nanoparticle-mediated gene transfection. Therefore, the aim of the current study was to investigate the critical formulation determinants with an objective to enhancing nanoparticle-mediated gene transfection.

MATERIAL AND METHODS

Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA) of different molecular weights (50:50 lactide/glycolide, Mw 12 kDa, 53 kDa, and 143 kDa), and composition (PLGA 75:25, PLGA 50:50, Mw 53 kDa) and Poly(D,L-lactide) (PLA, Mw 53 kDa) were purchased from Birmingham Polymers, Inc. (Birmingham, AL). Acetylated bovine serum albumin (Ac-BSA) and polyvinyl alcohol (PVA, average Mw 30–70 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO). PVA with different molecular weights (87–89% hydrolyzed, average Mw 13–23 kDa, 31–50 kDa, and 85–146 kDa) and degree of hydrolyzation (average Mw 9–10 kDa, 80% and 87–89% hydrolyzed) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 6-Coumarin was purchased from Polyscience Inc. (Warrington, PA). Fetal bovine serum (FBS, heat inactivated), 1× trypsin-EDTA, Rosewell Park Memorial Institute 1640 (RPMI 1640) medium, and penicillin-streptomycin were obtained from Gibco-BRL (Grand Island, NY). Human breast carcinoma (MCF-7) and human prostate cancer (PC-3) cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Luciferase plasmid with simian virus 40 (SV40) promoter and cytomegalovirus (CMV) enhancer (pGL3), cell culture lysis reagent (CCLR, 5×), luciferase assay kit, and the recombinant luciferase protein were purchased from Promega (Madison, WI). FuGENE™ 6 was purchased from Roche Diagnostics, Indianapolis, IN. Mitochondria/cytosol fractionation kit was purchased from BioVision Inc. (Mountain view, CA). All other chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Methods

Plasmid Preparation and Formulation of Nanoparticles Containing Plasmid DNA

Plasmid DNA encoding for luciferase gene (pGL3, with SV40 promoter and enhancer) was prepared using Qiagen® mega/giga column (Qiagen, CA). In brief, plasmid DNA was propagated in *E. coli* strain DH5α under defined growth conditions. The bacterial colonies containing plasmid DNA were selected, and DNA was extracted and purified using an ion-exchange resin column.

DNA-loaded nanoparticles were formulated using a double-emulsion solvent-evaporation technique as described by Prabha *et al.* (5). In brief, in a typical procedure, DNA solution (1 mg of DNA + 2 mg of nuclease-free acetylated BSA dissolved in 200 μl of TE buffer) was emulsified into a polymer solution (30 mg/ml) by sonication for 2 min using a probe sonicator at 55 W energy output (Sonicator® XL, Misonix, NY) to form a water-in-oil emulsion. Acetylated BSA was incorporated in the formulation because it is nuclease-free and hence is not expected to degrade DNA. BSA was used in the formulation to facilitate the release of DNA. The above emulsion was further emulsified into 6 ml of aqueous solution of PVA (concentration of PVA depends on the protocol) using the sonicator as above for 5 min to form a water-in-oil-in-water emulsion. The emulsion was stirred overnight to evaporate chloroform, and the nanoparticles thus formed were recovered by ultracentrifugation (35,000 rpm for 20 min at 4°C, Optima™ LE-80K, Beckman, Palo Alto, CA), washed twice to remove PVA and unentrapped DNA, resuspended in sterile water, and lyophilized for about 48 h.

For the cellular uptake studies, nanoparticles containing fluorescent marker in addition to DNA were prepared by dissolving 6-coumarin, a fluorescent dye (50 μg), in the polymer solution before emulsification. As reported in our previous studies, the incorporated dye acts a probe for nanoparticles without changing their physical properties (5).

Physical Characterization of Nanoparticles

Particle size and polydispersity of nanoparticles (0.5 mg/ml nanoparticles in distilled water) was determined using a Zeta Plus™ particle size analyzer (Brookhaven Instruments Corp., Holtsville, NY). The ζ potential (surface charge) of nanoparticles (0.5 mg/ml nanoparticles in distilled water) was determined using a Zeta Plus™ ζ potential analyzer. 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. The amount of DNA loaded in the nanoparticles was calculated from the standard curve of DNA prepared in washing solution obtained from control nanoparticles. The standard curve was prepared in the washing from the control nanoparticles so that BSA that is not encapsulated and is present in the washing does not interfere in the calculation of DNA concentration. The amount of PVA associated with nanoparticles was determined using a colorimetric method as described in our previous publication (11).

DNA release from nanoparticles under *in vitro* conditions was studied by incubating 0.15 mg of the respective formulation of nanoparticles 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). Previous studies have demonstrated that the DNA encapsulated and that released from nanoparticles maintained their conformation, suggesting that the homogenization conditions used in our studies do not cause fragmentation of DNA (5).

Transfection Studies and Determination of Luciferase Protein Levels

MCF-7 and PC-3 cells were grown in RPMI medium supplemented with 10% FBS, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. For transfection studies, cells were cultured at the seeding density of 35,000 cells/ml/well in 24-well plate 1 day before transfection. A nanoparticle suspension

was prepared in the serum-free medium (4 mg in 500 μl) using a water bath sonicator for 10 min (FS140, Fisher Scientific, Pittsburgh, PA). The nanoparticle suspension was then diluted to 9 ml with complete RPMI 1640 medium. The medium in the wells was replaced with 1 ml of nanoparticle suspension. Thus, the dose of nanoparticles per well was 444 μg/ml. The dose of nanoparticles used for the transfection was based on the preliminary dose–response study. Because the dose of nanoparticles was kept constant, the DNA dose per well varied and depended on the DNA loading in the respective formulation (Tables I and II). Medium was changed 1 day after the transfection with no further addition of nanoparticle dose. Medium was replaced on every alternate day thereafter. Cells were lysed at 1, 3, 5, and 7 days in the case of MCF-7 cells and at 3 days in the case of PC-3 cells. To measure luciferase protein levels, cells were washed twice using 1× phosphate-buffered 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 (pg/mg cell protein). Transfection studies with naked DNA (11.8 μg/ml/well) and naked DNA + PVA (PVA amount 16.7 μg/ml/well) were carried out as controls. The dose of PVA used for the above transfection study was calculated from the amount of PVA associated with the dose of nanoparticles used for transfection for the formulation prepared using 143-kDa polymer and 2% PVA solution as an emulsifier. Transfection with plasmid DNA (11.8 μg DNA/well/ml) using FuGENE™ 6 was carried out similar to that used for nanoparticles except that the transfection was carried out in the absence of serum as per the manufacturer's instructions. A 1:3 complex of plasmid DNA: FuGENE™ 6 was prepared in a serum-free medium, and the complex was then added to the cells. The medium was changed 1 day following the addition of the complex and on every alternate day thereafter.

Cellular Nanoparticle Uptake

For the cellular uptake study, a formulation of DNA-loaded nanoparticles containing 6-coumarin as a fluorescent marker was used. MCF-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. 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 lysate 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 (14,000 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 (12). A standard plot using different amounts of nanoparticles dispersed in 1× CCLR and treated similarly to cell lysate was used to quantify the nanoparticle levels. The uptake was represented as nanoparticle amount (μg/mg total cell protein).

Cell Fractionation

About 3×10^5 MCF-7 cells/well were seeded in six-well plates and incubated at 37°C, 5% CO_2 for 1 day. A suspension of nanoparticles containing fluorescent dye (888 μg/2 ml/well) was added to the cells and incubated at 37°C and 5% CO_2. The concentration of nanoparticle used in this study was the same as that used for the transfection studies. The medium was changed 1 day after incubation of cells with nanoparticles, and no further dose of nanoparticle was added. Medium was changed on every alternate day thereafter. At different time intervals (1 h, 8 h, 1 day, 3 days, 5 days, and 7 days), cells were trypsinized and harvested by spinning at 1000 rpm for 10 min, washed twice with 1× phosphate-buffered saline (PBS), and suspended in 1× cytosol extraction buffer containing dithiothreitol (DTT) and protease inhibitor cocktail (BioVision, Mountain View, CA). The cells were then homogenized using a pellet pestle® motor (Fisher) and centrifuged at 700 × *g* to collect the supernatant that contained the cytosol fraction. The supernatant was then lyophilized, and the dye from the nanoparticles was extracted using methanol as above. The samples were then analyzed for the nanoparticle levels using HPLC as described above. A standard curve was constructed using different concentrations of nanoparticles under identical conditions that were used for analysis of nanoparticle levels in cell lysate to quantify the amount of nanoparticles present in the cell fraction.

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).

RESULTS

Effect of Polymer Molecular Weight and Composition on Physical Properties and Gene Transfection of Nanoparticles

In general, DNA loading, intracellular uptake of vector, and DNA release from the vector are some of the factors that govern gene transfection mediated by polymeric nonviral gene delivery systems. Therefore, the DNA-loaded nanoparticles using polymers of different compositions and molecular weights were formulated and characterized for physical properties and gene transfection *in vitro*. Molecular weight of PLGA was found to affect the DNA entrapment in nanoparticles, with greater efficiency of DNA encapsulation observed in the nanoparticles formulated using higher-molecular-weight PLGA (143 kDa) (Table I). Particle size of the nanoparticles formulated using higher-molecular-weight polymer was smaller; the particles formed were more uniform in size as indicated by lower polydispersity index, and these particles had relatively lower negative ζ potentials as compared to those formulated using lower-molecular-weight polymers (Table I). The residual PVA associated with the nanoparticle

Table I. Physical Characterization of the Nanoparticles Formulated Using PLGA/PLA of Different Molecular Weights and Compositions

Formulation parameter	Polymer molecular weight (kDa)	Polymer composition (lactide: glycolide ratio)	PVA bound to nanoparticles (%w/w)*	Mean particle size (nm)*	Polydispersity index*	ζ potential (mV)**	DNA loading (mg/100 mg nanoparticles)	DNA dose (μ/ml/well) used for transfection
Effect of molecular weight of PLGA	12	50:50	2.3 ± 0.9	563 ± 6	0.25 ± 0.02	−17.8 ± 1.0	1.8	8.0
	53	50:50	2.2 ± 0.3	685 ± 40	0.32 ± 0.02	−16.6 ± 1.4	1.7	7.5
	143	50:50	3.8 ± 0.4	375 ± 22	0.19 ± 0.01	−11.5 ± 3.4	2.9	12.9
Effect of polymer composition	53	100:0	2.4 ± 0.3	571 ± 9	0.21 ± 0.02	−14.3 ± 1.7	1.8	8.0
	53	75:25	2.2 ± 0.2	485 ± 11	0.23 ± 0.01	−16.6 ± 1.4	1.9	8.4
	53	50:50	2.2 ± 0.3	685 ± 40	0.32 ± 0.03	−18.2 ± 3.8	1.7	7.5

* Data represented as mean ± SEM, *n = 3, **n = 5.

Table II. Physical Characterization of the Nanoparticles Formulated Using Different PVA

Formulation parameter	PVA concentration (%)	PVA average molecular weight (kDa)	PVA degree of hydrolyzation (%)	PVA bound to nanoparticles (%w/w)*	Mean particle size (nm)*	Polydispersity index*	ζ potential (mV)**	DNA loading (mg/100 mg nanoparticles)	DNA dose (μ/ml/well) used for transfection
Effect of PVA concentration	0.5	31–50	88	2.2 ± 0.2	693 ± 2	0.3 ± 0.04	−31.3 ± 1.6	1.1	4.8
	2.0	31–50	88	3.8 ± 0.4	347 ± 3	0.2 ± 0.02	−11.5 ± 3.4	2.9	12.9
	5.0	31–50	88	5.2 ± 0.7	270 ± 1	0.2 ± 0.01	−6.5 ± 1.7	2.9	12.9
Effect of PVA molecular weight	2.0	13–23	88	5.7 ± 0.2	561 ± 22	0.17 ± 0.02	−15.6 ± 5.8	2.4	10.7
	2.0	31–50	88	4.1 ± 0.3	560 ± 3	0.21 ± 0.04	−17.3 ± 5.3	1.8	8.0
	2.0	83–146	88	3.7 ± 0.6	1207 ± 30	0.26 ± 0.04	−14.9 ± 4.7	1.9	8.4
Effect of PVA degree of hydrolyzation	2.0	13–23	80	9.1 ± 0.9	344 ± 30	0.21 ± 0.01	−22.2 ± 1.8	1.3	5.8
	2.0	13–23	89	5.7 ± 0.2	561 ± 22	0.17 ± 0.02	−15.6 ± 5.8	2.4	10.7

* Data represented as mean ± SEM, *n = 3, **n = 5.

surface was higher for the nanoparticles formulated using higher-molecular-weight PLGA (Table I). Furthermore, the DNA release from the nanoparticles formulated using higher-molecular-weight polymer was relatively higher than that from the nanoparticles formulated using lower-molecular-weight polymers (Fig. 1a). Transfection studies demonstrated that gene expression of nanoparticles increased with an increase in molecular weight of polymer used in their formulation. Nanoparticles formulated using PLGA of molecular weight 143 kDa demonstrated 50- to 100-fold, and the nanoparticles formulated using 53-kDa polymer demonstrated approximately 6- to 15-fold greater gene transfection, in comparison to the nanoparticles formulated using 12-kDa molecular weight polymer in MCF-7. In addition to the particle size, molecular weight of the polymer seems to play a role because the nanoparticles formulated with 53-kDa and 12-kDa polymers have similar particle size, DNA loading, and ζ potential, but the transfection with nanoparticles formulated with higher-molecular-weight polymer was higher than that of nanoparticles formulated with lower-molecular-weight polymer. Similar relatively higher gene transfection was observed in PC-3 cells for the nanoparticles formulated using higher-molecular-weight polymer than the gene transfection with the nanoparticles formulated using lower-molecular-weight polymers (Fig. 1c).

Gene transfection with an equivalent dose of naked DNA was about 50-fold lower than the gene transfection with the nanoparticles formulated using 143-kDa molecular weight PLGA in MCF-7 cells (Fig. 1b). Gene transfection with FuGENE™ 6, a commercially available transfection agent, was relatively higher than the gene transfection with nanoparticles in MCF-7 cells; however, the transfection level declined almost exponentially with time in case of the transfection agent (Fig. 2), whereas the level remained sustained with nanoparticles (Fig. 1b).

In further studies, the effect of polymer composition (lactide to glycolide ratio) on nanoparticle characteristics and gene transfection was studied. Nanoparticles were formulated using PLA (100% lactide) and PLGA of different compositions (lactide to glycolide ratio 75/25, 50/50) having an average molecular weight of about 53 kDa. Although the polymer composition did not show significant differences in the physical characteristics of nanoparticles (Table I), the cumulative DNA release from the nanoparticles formulated using PLA was relatively lower than that from the other two formulations of nanoparticles (Fig. 3a). Nanoparticles formulated using PLA demonstrated significantly lower gene transfection as compared to gene transfection with the nanoparticles formulated using polymers containing glycolide in part (PLGA) in both MCF-7 (Fig. 3b) and PC-3 (Fig. 3c) cells.

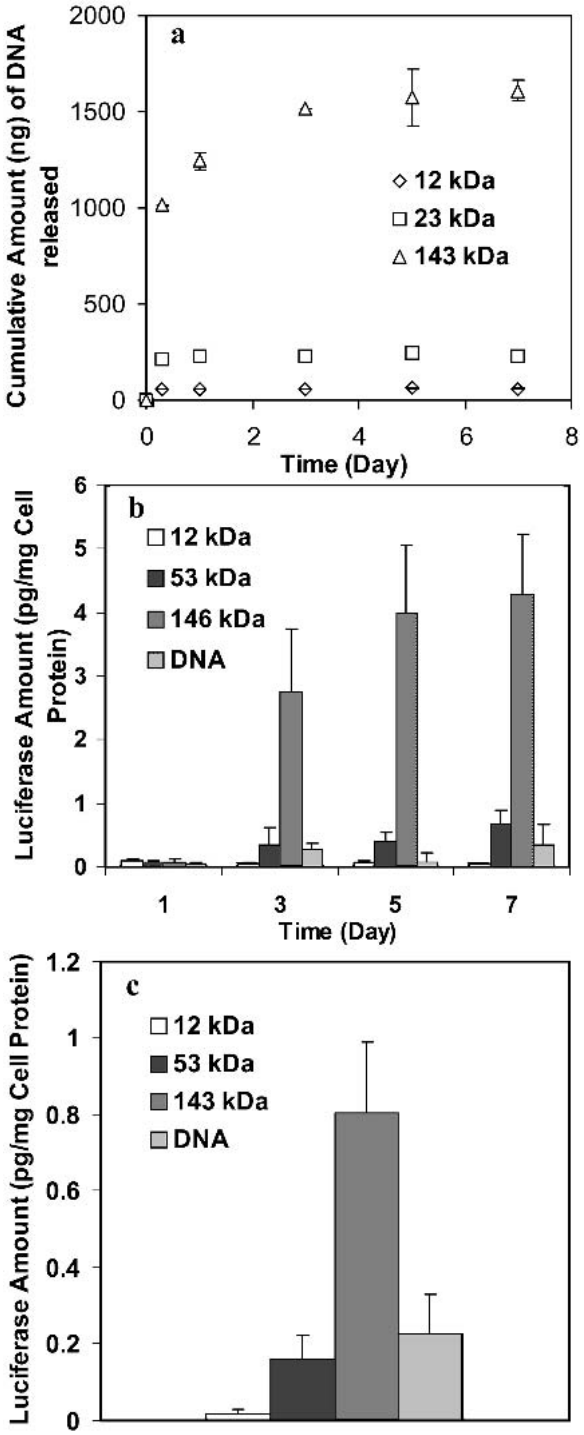

Fig. 1. Effect of molecular weight of PLGA on (a) *in vitro* release of DNA from nanoparticles and transfection of nanoparticles in (b) MCF-7 and (c) PC-3 cells. Cells (35,000 per well in 24-well plate) were incubated with nanoparticles (444 μg/ml/well, see Table I for dose of DNA) for 1 day after which the medium in the wells was replaced with fresh medium (without nanoparticles). Medium was changed on alternate days thereafter. Nanoparticles showed sustained gene transfection in MCF-7 cell line. In PC-3 cells, transfection was determined at the end of 3 days postincubation with nanoparticles. Studies in PC-3 could not be continued beyond 3 days because the cells reached confluency. Figure legend represents molecular weight of PLGA. Data as mean ± SEM, n = 6.

Effect of Emulsifier (PVA) Concentration on Physical Properties, Transfection, Cellular Uptake, and Intracellular Distribution of Nanoparticles

Nanoparticle surface characteristic is a critical determinant because it determines the interaction of nanoparticles with the cell surface. It has been known that a fraction of PVA remains associated with PLGA and PLA nanoparticles and forms an interface (11,13). In order to study the effect of surface-associated PVA on gene transfection, nanoparticles (PLGA 50/50, molecular weight 143 kDa) were formulated

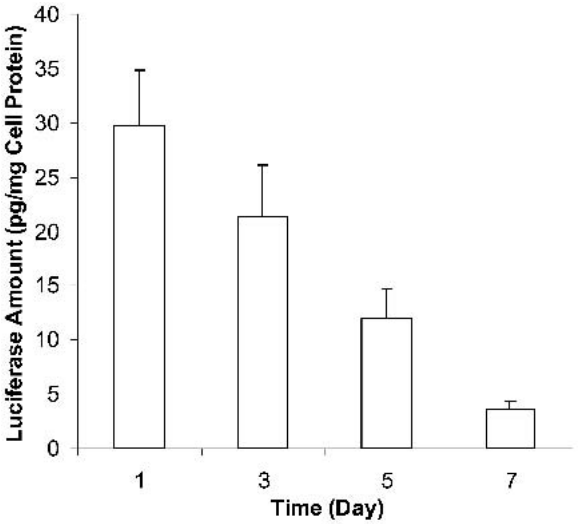

Fig. 2. Transfection with FuGENE™ 6. Plasmid DNA: FuGENE™ 6 (1:3) complex (DNA dose ~11.8 μg/ml/well) was prepared in serum-free medium, and the complex was added onto the cells (35,000 cells/well in 24-well plate). The medium in the wells was changed to the regular serum-containing medium at 24 h, and the luciferase protein levels were analyzed at the end of 1, 3, 5, and 7 days posttransfection. Data represented as mean ± SEM, n = 6.

using PVA (average molecular weight 31–50 kDa and 89% degree of hydrolyzation) at different concentrations. Although the particle size was reduced with the increase in PVA concentration, the nanoparticle-surface-associated PVA increased (Table II). PVA bound to the nanoparticle surface was found to affect the interfacial properties of nanoparticles, especially the surface charge, since the nanoparticles with higher amount of surface associated PVA had reduced anionic charge (Table II). DNA loading in the nanoparticles formulated with lower concentration of PVA (0.5% w/v) was lower than that in the nanoparticles formulated using 2% and 5% w/v PVA (Table II).

To study the effect of surface-associated PVA on gene expression, nanoparticles formulated using 2% w/v and 5% w/v PVA concentration were used for comparison as these formulations had almost similar DNA loading and particle size. Nanoparticles with lower amounts of surface-associated PVA demonstrated 12- to 20-fold higher gene transfection in MCF-7 cells than those with higher amount of surface-associated PVA (Fig. 4b). Similar higher transfection was observed in PC-3 cell line for the nanoparticles formulated using 2% w/v PVA; however, the difference in the transfection was only twofold in this cell line (Fig. 4c).

Despite relatively lower DNA loading in the nanoparticles formulated with 0.5% w/v PVA than that in the nanoparticles formulated with 5% w/v PVA, gene transfection of the nanoparticles formulated with 0.5% w/v PVA was 1.5- to 3-fold higher as compared to the gene transfection of the nanoparticles formulated with 5% PVA in MCF-7 cells. However, the gene transfection of the nanoparticles formulated with 2% w/v PVA was 10- to 20-fold greater than the gene transfection of the nanoparticles formulated with 5% PVA despite similar DNA loading. To demonstrate that the difference in the gene expression observed with different formulations of nanoparticles was caused by the effect of surface-

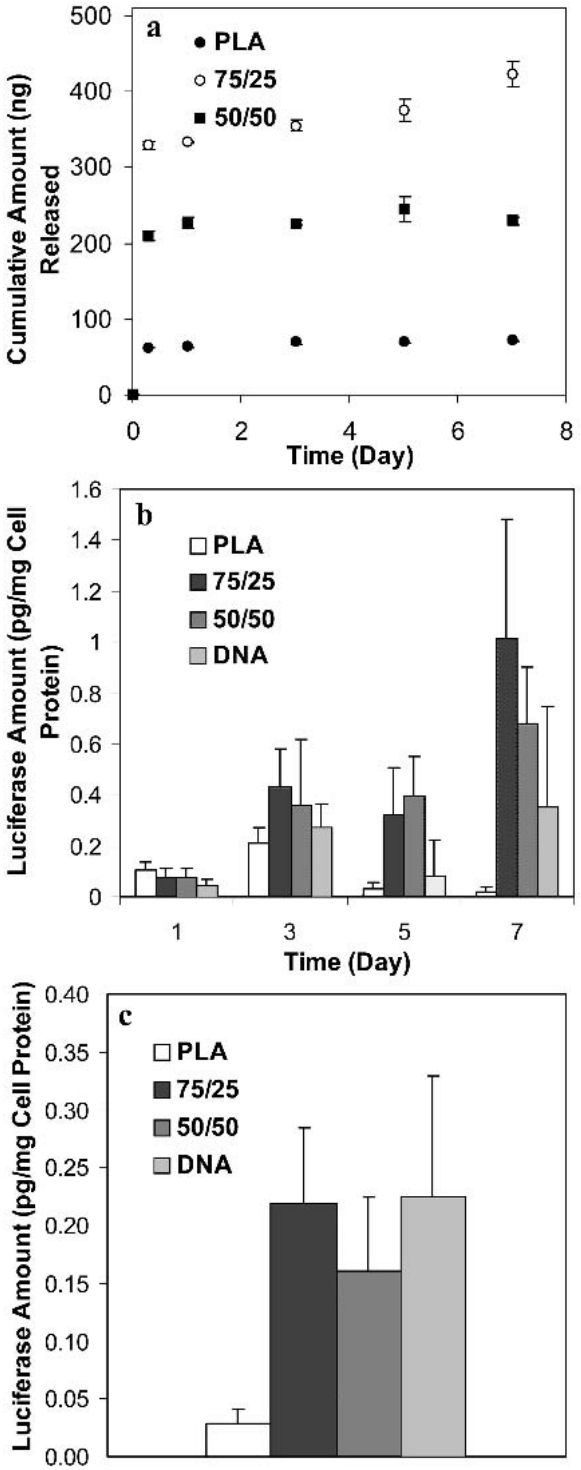

Fig. 3. Effect of polymer composition on (a) *in vitro* DNA release from nanoparticles and transfection of nanoparticles in (b) MCF-7 and (c) PC-3 cells. Cells (35,000/well in 24-well plate) were incubated with nanoparticles (444 μg/ml/well) for 1 day, and then the medium was replaced with fresh medium (without nanoparticles). Medium was changed on every alternate day thereafter, and transfection levels were determined at 1, 3, 5, and 7 days posttransfection in MCF-7 cell line and at 3 days posttransfection in PC-3 cell line. Figure legend represents lactide:glycolide ratio. Data shown as mean ± SEM, n = 6.

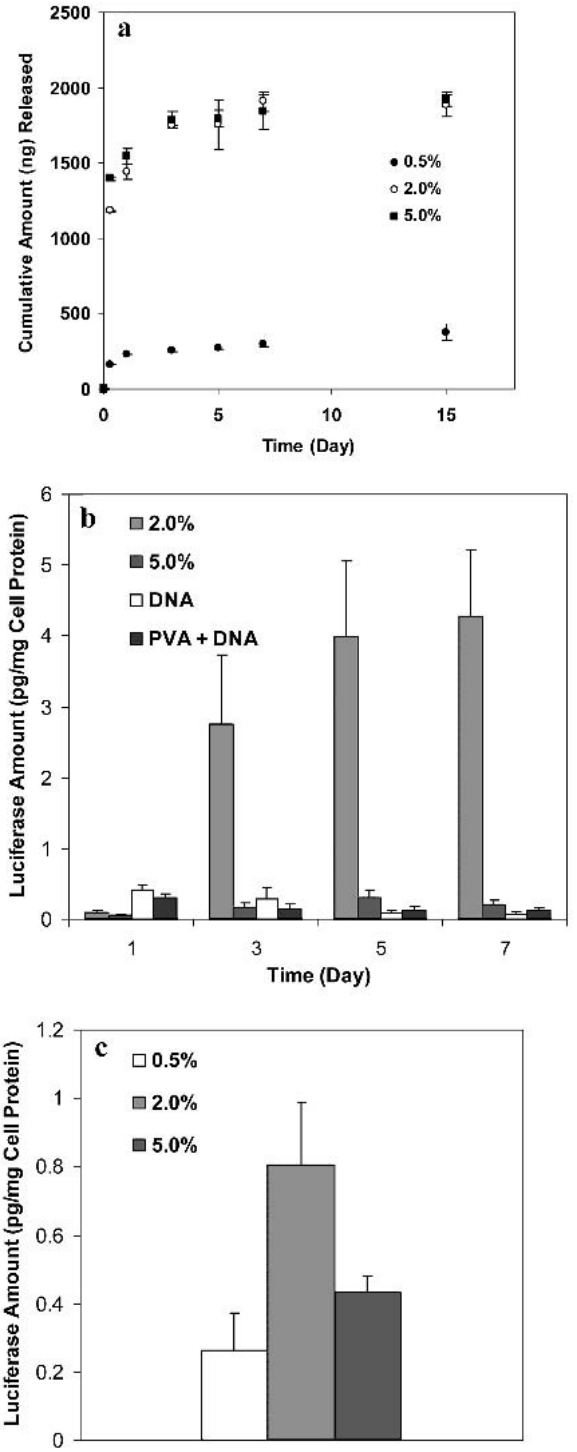

Fig. 4. Effect of PVA concentration on (a) *in vitro* release of DNA from nanoparticles and transfection of nanoparticles in (b) MCF-7 and (c) PC-3 cells. Cells (35,000 per well in 24-well plate) were incubated with nanoparticles (444 μg/ml/well, see Table II for dose of DNA) for 1 day, and then the medium in wells was replaced with fresh medium (without nanoparticles). Medium was changed on every alternate day thereafter, and luciferase protein levels were determined at 1, 3, 5, and 7 days posttransfection in MCF-7 cell line and at 3 days posttransfection in PC-3 cell line. Figure legend represents lactide:glycolide ratio. Identical protocol was used to determine transfection of plasmid DNA or plasmid DNA + PVA. Data shown as mean ± SEM, n = 6. Figure legend represents concentration of PVA used as an emulsifier. Data shown as mean ± SEM, n = 6.

associated PVA, a control experiment with plasmid DNA and plasmid DNA mixed with PVA was carried out. There was no significant difference in the transfection levels of DNA and DNA + PVA ($p > 0.05$) (Fig. 4b).

In order to account for the difference in the transfection of the two formulations of nanoparticles (prepared using 2% and 5% w/v PVA) despite similar DNA loading and release (Fig. 4a), the cellular uptake of the above two formulations of nanoparticles was determined. The results demonstrated a 1.5-fold higher uptake for the nanoparticles prepared using 2% w/v PVA as compared to that for the nanoparticles formulated using 5% w/v PVA (26 ± 1.4 μg nanoparticles/mg cell protein for 2% w/v PVA vs. 17.5 ± 1 μg nanoparticles/mg cell protein for 5% w/v PVA). Further analysis of the intracellular distribution of nanoparticles demonstrated that the nanoparticles formulated with 2% w/v PVA had over two-fold higher cytoplasmic levels than the nanoparticle levels for those formulated with 5% w/v PVA (Fig. 5). The cytoplasmic nanoparticle levels increased with incubation time, but these levels dropped gradually once the medium was changed after 1 day.

Effect of PVA Molecular Weight and Degree of Hydrolyzation on Physical Properties and Transfection of Nanoparticles

Because the surface-associated PVA was found to have a significant effect on the transfection of nanoparticles, further studies were carried out with the nanoparticles formulated using PVA of different molecular weights and degrees of hydrolyzation. In initial studies, nanoparticles were formulated (PLGA 50/50, molecular weight 143 kDa) using PVA of molecular weight 13–23 kDa but differing in the degree of hydrolyzation (80% and 89%). With the increase in degree of hydrolyzation of PVA, the DNA loading in nanoparticles increased (Table II), the surface-associated PVA was reduced, and gene transfection of nanoparticles was enhanced (Fig. 6a). Furthermore, the amount of DNA released from the nanoparticles formulated using PVA with higher (89%) degree of hydrolyzation was relatively greater than that from the nanoparticles formulated using PVA with lower (80%) degree of hydrolyzation (640 ± 40 ng vs. 360 ± 40 ng cumulative DNA release at the end of 7 days).

In a second set of experiments, nanoparticles were formulated (PLGA 50/50 of molecular weight 143 kDa) using PVA with the same degree of hydrolyzation (89%) but differing in molecular weight. Nanoparticle size was greater for the formulation prepared with high-molecular-weight PVA as compared to that prepared using lower-molecular-weight PVA (Table II). Also, the nanoparticles formulated with lower-molecular-weight PVA had relatively higher DNA loading. Despite relatively higher DNA loading and similar DNA release (643 ± 78 ng cumulative DNA release at the end of 7 days for 13- to 23-kDa PVA vs. 689 ± 27 ng for 31- to 50-kDa PVA vs. 636 ± 30 ng for 85- to 146-kDa PVA), the nanoparticles formulated using PVA of lower molecular weight (13–23 kDa) showed lower transfection levels than those formulated using PVA of molecular weight 31–50 kDa and 85–146 kDa (Fig. 6b).

DISCUSSION

Gene expression using nonviral vectors depends on several factors including efficient intracellular uptake of the ex-

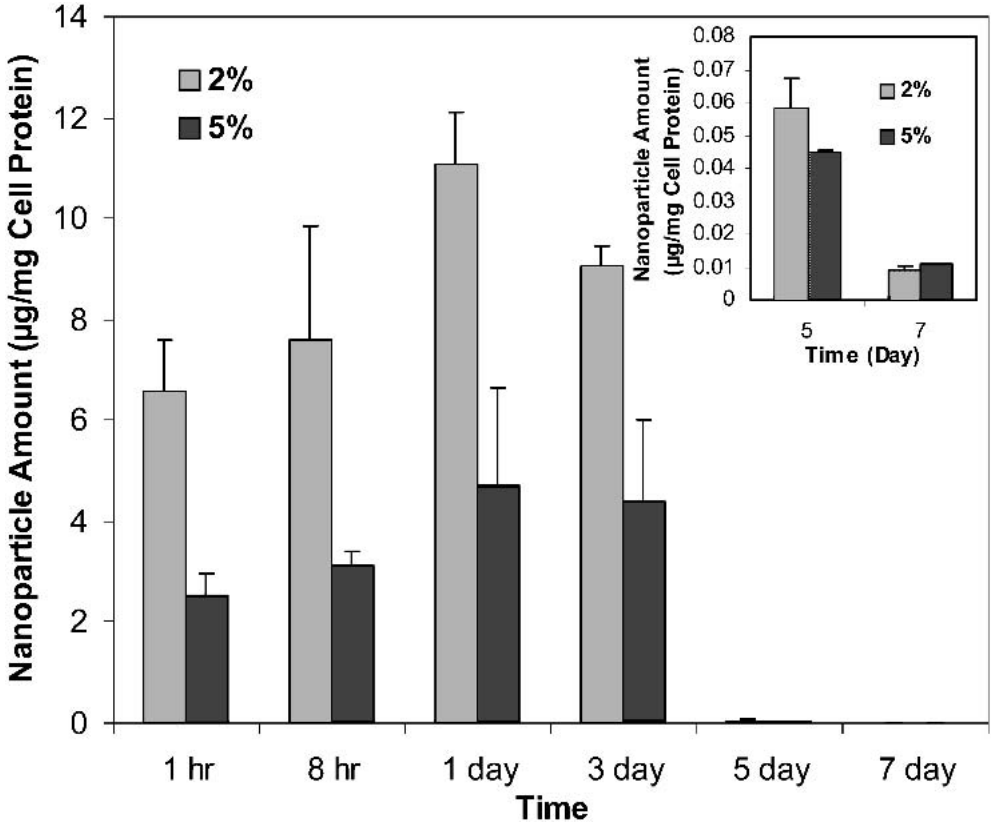

Fig. 5. Cytoplasmic levels of nanoparticles formulated using different concentrations of PVA in MCF-7 cells. Cells (3×10^5 in 6-well plates) and the nanoparticle levels in the cytoplasm were determined at 1 h, 8 h, and 1 day postincubation. Medium was changed at 1 day with no further addition of nanoparticles and on every alternate day thereafter. Cytoplasmic nanoparticle levels were then determined at 3, 5, and 7 days postnanoparticle incubation. Inset shows the cytoplasmic levels of nanoparticles at 5 and 7 days of post-nanoparticle incubation. Data shown as mean ± SEM, n = 6.

pression vector, their escape from the degradative environment inside the endolysosomal compartment, dissociation of DNA from the vector, and the effective localization of DNA into the nucleus (3). Although different strategies are being investigated to overcome the barriers associated with gene delivery, the efficiency of gene expression with nonviral vectors remains relatively lower (14). Among several nonviral vectors, cationic polymers and lipid–DNA complexes are relatively more efficient; however, toxicity concerns and instability of these systems in the presence of serum limit their effective use for *in vivo* applications (14,15). Different approaches are being investigated to overcome the problems associated with the above systems (16,17).

Nanoparticles and microparticles formulated using PLGA and PLA polymers are recently being investigated as a nonviral gene delivery system because of their sustained release characteristics, biocompatibility, and biodegradability, and their ability to protect DNA from degradation in endolysosomes (4–6,18). Although PLGA/PLA nanoparticles are extensively investigated for drug and protein delivery (19), their application as a gene expression vector is recent. Our recent studies demonstrated that nanoparticles are internalized efficiently into cells, following which a fraction of them rapidly escapes the endolysosomes into the cytoplasm compartment (6). Escape of the expression vector from the endolysosomal compartment is an important characteristic because most of the DNA degrades rapidly in this compartment (20).

In our studies, nanoparticle-mediated gene transfection increased with the increase in molecular weight of polymer, which could be explained based on relatively higher DNA loading and its release from the nanoparticles formulated with higher-molecular-weight polymer. Higher DNA loading in these nanoparticles could be related to the higher viscosity and better emulsifying properties of the polymer solution. This could have resulted in the formation of a more stable emulsion and, therefore, lower diffusion of DNA from the particles during the formulation step (21). It has been shown previously that the increase in the viscosity of the oil phase in the multiple emulsion leads to restricted movement of the water droplets inhibiting droplet coalescence and DNA loss that occur during the formation of secondary emulsion (21–23). The better emulsifying properties of higher-molecular-weight polymer are also evident from the lower particle size and more uniform particle size distribution data (Table I). Higher DNA release from the nanoparticles formulated using higher-molecular-weight PLGA could be explained based on the higher DNA loading in these nanoparticles (24). Higher DNA loading in nanoparticles probably leads to the formation of pores and channels as DNA is released initially, leading to further release of DNA through the channels formed. Thus, the relatively higher gene transfection observed with

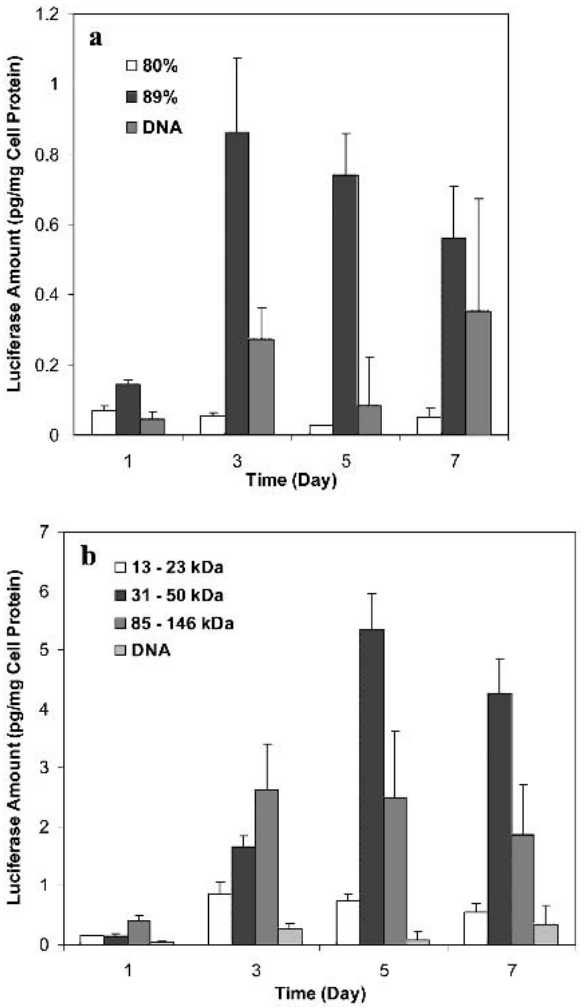

Fig. 6. Effect of (a) degree of hydrolyzation and (b) molecular weight of PVA used in the formulation of nanoparticles on transfection in MCF-7 cell line. Cells (35,000 per well in 24-well plate) were incubated with nanoparticles (444 μg/ml/well, see Table II for dose of DNA) for 1 day, and then medium was replaced with fresh medium (without nanoparticles). Medium was changed on every alternate day thereafter, and luciferase protein levels were determined at 1, 3, 5, and 7 days posttransfection in MCF-7 cell line. Data shown as mean ± SEM, n = 6.

the nanoparticles formulated with higher-molecular-weight polymer is related to the greater amount of DNA available to the cells for transfection.

Higher gene expression observed with 143-kDa PLGA nanoparticles as compared to plasmid DNA suggests that nanoparticles probably facilitate the internalization of DNA as well as protect it from degradation during their passage through the endolysosomal compartment into the cytoplasm. Sustained gene expression observed with nanoparticles suggests that the DNA is released slowly from the nanoparticles localized in the cytoplasmic compartment, which is then localized into the nucleus. In contrast, the gene expression with FuGENE™ 6, a commercially available transfection reagent (for *in vitro* transfection only), rapidly declines with time (Fig. 2), thus confirming the ability of nanoparticles to sustain gene expression. Sustained gene transfection achieved with nanoparticles could be beneficial in chronic disease conditions that require low levels of protein expression for longer intervals of time (25).

Polymer composition was also found to affect the transfection properties of nanoparticles. In general, polymers with a higher proportion of lactic acid are more hydrophobic than those with a higher fraction of glycolic acid. Nanoparticles formulated from polymer containing only lactide (polylactides) demonstrated lower transfection than those formulated using copolymers containing glycolide. This could again be explained based on the lower DNA release from the polylactide nanoparticles (Fig. 3a), which was probably related to the lower diffusion of DNA through the highly hydrophobic polymer matrix and also lower degradation rate of the hydrophobic polymer.

PVA is a commonly used emulsifier in the formulation of nanoparticles, mainly because the nanoparticles formed are smaller and uniform in size and are easy to redisperse in buffer or saline. It has been shown in our studies and also by others that a fraction of PVA remains associated with the nanoparticle surface even after multiple washings (13). We have previously shown that this residual PVA affects the interfacial characteristics of nanoparticles and also their cellular uptake (11). Hence, we hypothesized that the change in the interfacial properties of nanoparticles as a result of the surface-associated PVA could affect the nanoparticle-mediated gene transfection. The lower concentration of PVA (0.5% w/v) used as an emulsifier resulted in nanoparticles with lower DNA loading and larger size (Table II), but nanoparticles formulated using 2% w/v and 5% w/v PVA solution were smaller in size and had higher DNA loading than those formulated with 0.5% PVA. This could be because of the stabilizing effect of PVA on the emulsion, leading to greater entrapment of DNA into nanoparticles and smaller particle size. Because nanoparticles formulated using 0.5% w/v PVA were different in terms of DNA loading, only the nanoparticles formulated using 2% w/v and 5% w/v PVA can be used to compare the effect of nanoparticle surface-associated PVA on gene transfection. Although there was no difference in the DNA loading and release from nanoparticles formulated with 2% w/v and 5% w/v PVA, the difference in the transfection between the two formulations of nanoparticles was significant ($p = 0.001$, day 5 and day 7). Nanoparticles formulated with the lower concentration of PVA (2% w/v) demonstrated 12- to 20-fold higher transfection in MCF-7 than the nanoparticles formulated with higher-concentration PVA (5% w/v) (Fig. 4b). Cellular uptake studies demonstrated that nanoparticles formulated with higher concentration of PVA have reduced cellular uptake, and these results are consistent with our previously reported studies in vascular smooth muscle cells (11). Further analysis of intracellular distribution demonstrated that the nanoparticles formulated with lower concentration of PVA had a greater amount of nanoparticles in the cytoplasmic fraction than the nanoparticles formulated with a higher concentration of PVA. The difference in nanoparticle levels in the cytoplasmic fraction thus could explain the difference in the transfection levels between the two formulations of nanoparticles (2% w/v and 5% w/v PVA). Higher intracellular uptake and cytoplasmic levels of the nanoparticles formulated using 2% w/v PVA could be related to their surface charge. In our previous studies, we have shown that the surface charge reversal of the nanoparticle in the acidic pH usually found in the endolysosomes is the

mechanism of endosomal escape of nanoparticles (6). The shielding of the surface charge reversal by the presence of a higher amount of surface-associated PVA in nanoparticles (11) could affect their escape from the endolysosomal compartment to the cytoplasmic compartment.

The study also demonstrated that not only the concentration of PVA but also the type of PVA (molecular weight and degree of hydrolyzation) used in nanoparticle formulation influences gene transfection. Nanoparticles formulated using low-molecular-weight PVA demonstrated lower gene transfection compared to those formulated using higher-molecular-weight PVA despite higher DNA loading and similar DNA release from these particles. This could be related to the higher amount of residual PVA associated with the nanoparticles formulated with low-molecular-weight PVA than those formulated using higher-molecular-weight PVA (Table II). Similarly, the nanoparticles formulated using PVA of higher degree of hydrolyzation had a reduced amount of surface-associated PVA. These results are similar to those reported previously, where PLGA nanoparticles prepared by the spontaneous emulsification solvent diffusion method had a higher amount of PVA bound when PVA of lower degree of hydrolyzation was used (26). Relatively lower amounts of surface-associated PVA, higher DNA loading, and greater DNA release from the nanoparticles formulated using PVA of higher degree of hydrolyzation could explain the higher gene transfection of these nanoparticles as compared to gene transfection of the nanoparticles formulated using PVA of lower degree of hydrolyzation. Some of the differences in transfection seen with various formulations of nanoparticles prepared with different PVA could also be related to the difference in their sticking property to the cell surface through the surface-associated PVA. However, recently in rabbit conjunctival epithelial cells, we have demonstrated that about 90% of the particles associated with cells are internalized, and the remaining fraction (10%) is surface associated (unpublished results). Therefore, the influence of PVA affecting the gene expression of nanoparticles through the difference in their sticking properties to the cell surface could be marginal and appears to be mainly linked to the difference in their uptake and intracellular distribution.

Based on the influence of surface-associated PVA on gene transfection of nanoparticles, it could be speculated that the stealth nanoparticles or liposomes that are used to achieve prolonged systemic circulation would have reduced gene expression in the target cells or tissue as compared to that with nonstealth systems. In fact, Shi *et al.* (27) have demonstrated that inclusion of poly(ethylene glycol)–lipid analogues in oligonucleotide (ODN) lipoplex inhibited their internalization in Chinese hamster ovary cells by more than 70%. Furthermore, they observed that the intracellular fraction of lipoplex remained entrapped in the endolysosomal pathway, and no release of ODNs was seen.

Although we have not studied the effect of different formulation parameters on gene transfection of nanoparticles *in vivo*, it seems logical to believe that the parameters investigated in this study that influenced the DNA loading in nanoparticles, DNA release from nanoparticles, and cellular uptake of nanoparticles would also affect the gene expression of nanoparticles *in vivo*. Therefore, the conclusions drawn from the *in vitro* studies in this paper about nanoparticle formulation parameters are critical in achieving higher gene transfection *in vivo* using nanoparticles. Unlike polyplexes and lipoplexes, nanoparticles are anionic in physiologic pH and do not aggregate under the physiologic conditions or in the presence of serum. It is to be noted that all the transfection studies in this paper with nanoparticles were carried out in the presence of serum. Therefore, nanoparticles can be used *in vivo* to achieve sustained gene transfection.

CONCLUSIONS

In conclusion, various formulation factors were found to affect the nanoparticle-mediated gene transfection. Polymer characteristics such as composition and molecular weight influenced gene transfection mainly through their effect on DNA release from nanoparticles. Emulsifier characteristics influenced gene transfection through their effect on cellular uptake, endolysosomal escape, and/or DNA release. Although some of the formulation factors are interconnected, nanoparticles formulated from polymer composed of 50/50 lactide:glycolide and of high molecular weight (143 kDa) and with 2% w/v PVA (88% hydrolysis and average molecular weight of 31–50 kDa) demonstrated greater gene transfection than the other formulations investigated in this study.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support from the Nebraska Research Initiative, Gene Therapy Program. S.P. is supported by a predoctoral fellowship from the Department of Defense, US Army Medical Research and Materiel Command (DAMD17-02-1-0506). We would also like to thank Ms. Elaine Payne for providing administrative support.

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