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Poly(L-lactic acid)/polyethylenimine Nanoparticles as Plasmid DNA Carriers

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Non-viral vectors such as liposomes, polycations, and nanoparticles have been used as gene delivery systems. In this study, we prepared and characterized biodegradable poly(L-lactic acid) (PLA)/polyethylenimine (PEI) nanoparticles as gene carriers. pCMV/β-gal and pEGFP-C1 were utilized as model plasmid DNAs (pDNA). Nanoparticles were prepared using a double emulsion-solvent evaporation technique, and their pDNA binding capacity was assessed by agarose gel electrophoresis. Transfection was studied in HEK 293 and HeLa cell lines, and the transfection efficiencies were determined by β-galactosidase assay or flow cytometry. Three kinds of PLA/PEI systems were studied by varying the molecular weight of PEI. The PLA/PEI 25K system had a higher transfection efficiency than the PLA/PEI 0.8K or PLA/PEI 750K systems. The transfection efficiency was found to be dependent on the ratio of PLA/PEI nanoparticles to pDNA with an optimum ratio of 60:1 (w/w). The cytotoxicity was dependent on the quantity of PLA/PEI nanoparticles used, but it was comparable to that of commercial Lipofectin™. These results demonstrate the potential of PLA/PEI nanoparticles as gene carriers.

Key words: Nanoparticles, Poly(L-lactic acid), Polyethylenimine, Transfection, Non-viral gene delivery

INTRODUCTION

Non-viral gene delivery systems have been developed for gene therapy using cationic lipids, polycationic polymers (Choi *et al*., 1998; Kabanov *et al*., 2002), polymeric micelles (Kakizawa and Kataoka, 2002) and nanoparticles (Rhaese *et al*., 2003; Tiyaboonchai *et al*., 2003). These non-viral vectors have several advantages, including ease of manipulation, stability, low cost, safety, and high flexibility regarding the size of transgene delivery (Kircheis et al., 2001). Long-term gene expression has been achieved in polymer-mediated systems, and biodegradable nanoparticles can be used to deliver drugs and genes due to their safe and sustained actions (Prabha *et al*., 2002). Other merits of nanoparticles include their high stability, easy uptake into cells by endocytosis and ability to target specific tissues or organs via their surface adsorption or by coating with ligand materials (Lobenberg *et al*., 1997).

Two kinds of nanoparticles carrying pDNA are known, the DNA entrapment system (Hirosue *et al*., 2001; Leong *et al*., 1998; Perez *et al*., 2001) and the DNA surface binding system (Bivas-Benita *et al*., 2004; Ravi-Kumar *et al*., 2004). The DNA entrapment system is more common system and involves the encapsulation of pDNA. This system has the advantages of pDNA protection and controlled release of the encapsulated pDNA. The surface binding system utilizes ionic interactions between cationic polymers and pDNA. Cationically surface modified nanoparticles can readily bind and condense pDNA. Using such a system, the direct contact between pDNA and organic solvents can be avoided during nanoparticle preparation.

PEI has been extensively used as a non-viral DNA delivery system in various cells, and enables efficient gene transfer without the need of other endosomolytic agents (Brown *et al*., 2001). Moreover, the condensed PEI/DNA particles have been shown to be phagocytosed and to enter cellular endosomal and lysosomal compartments (Zheng *et al*., 1998).

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In the present study, we describe the development of a biodegradable nanoparticle system composed of polymer blends, specifically the mixtures of poly(L-lactic acid) (PLA) and polyethylenimine (PEI) that can carry pDNA (Kim *et al*., 2005). PLA/PEI nanoparticles were prepared and evaluated as non-viral gene carriers using pCMV/β-gal, a mammalian expression vector for β-galactosidase and pEGFP-C1 expressing green fluorescent protein as model pDNAs. In addition, the effect of PEI on the physicochemical characteristics of nanoparticles and its interaction with pDNA were studied for various molecular weights of PEI. Furthermore, we determined the optimum conditions required for maximum transfection efficiency and minimum cell cytotoxicity.

MATERIALS AND METHODS

Materials

PLA (Mw=50000) was purchased from Polysciences Inc. (Warrington, PA). PEIs (Mw=0.8K, 25K and 750K, branched) and polyvinyl alcohol (PVA) (87-89% hydrolyzed, Mw=13-23K) were purchased from Aldrich (St. Louis, MO). pCMV/β-gal (7.2 kb) and pEGFP-C1 (4.7 kb) were obtained from Clontech (Palo Alto, CA). Minimum essential medium, Dulbecco's modified Eagle's medium (DMEM) and Lipofectin™ were products of Gibco-BRL (Gaithersburg, MD). Fetal bovine serum (FBS), penicillin-streptomycin solution, agarose, Tris-EDTA (TE) buffer and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma Chemical Co. (St. Louis, MO). All solvents were HPLC grade and other reagents were analytical grade.

Amplification and purification of pDNA

pDNA was transformed into *E. coli DH 5α* bacterial strain. Transformed cells were grown in autoclaved LB broth (10 g tryptone, 5 g yeast extract and 10 g NaCl dissolved in 1 L water) containing appropriate antibiotics. pDNA was purified using a plasmid midi kit (Qiagen, Valencia, CA) and the purified pDNA was diluted in TE buffer. pDNA concentrations were measured by UV absorption at 260 nm, and the purity was confirmed using the Abs (260 nm)/Abs (280 nm) ratio and 1% agarose gel electrophoresis.

Preparation of PLA/PEI nanoparticles

Nanoparticles were prepared using a double emulsion-solvent evaporation technique (Zambaux *et al*., 1998). First, 4 mL PLA solution (25 mg/mL in methylene chloride) was mixed with aqueous PEI solution (5 mg/mL), and then 16 mL 5% PVA was added and sonicated to produce a w/o/w emulsion. The solvent was then evaporated under vacuum using a rotary evaporator at room temperature for 30 min. PLA/PEI nanoparticles were recovered by centrifugation at 17000 rpm, washed three times with distilled water, and lyophilized. DNA-loaded nanoparticles were prepared by gently mixing the aqueous suspension of PLA/PEI nanoparticles (10-500 μg/mL) with pCMV/β-gal or pEGFP-C1 (2 μg) at room temperature for 20 min.

Particle size and zeta-potential

Nanoparticle sizes and size distributions were determined by particle size analyzer (ELS8000, Otsuka Electronics, Japan). Nanoparticle samples were analyzed for at least three samples (10 readings/sample) after dispersion in distilled water at 25°C.

The zeta-potentials of the prepared nanoparticles were determined using a zeta-potential analyzer (ELS8000, Otsuka Electronics, Japan). Briefly, 10 mg of a nanoparticle sample was dispersed in 5 mL phosphate-buffered saline (PBS) and zeta-potential was measured at 25°C. The zeta-potentials of pDNA-adsorbed nanoparticles were determined using the same method.

Gel electrophoresis of the pDNA complex

The electric mobilities of the pDNA complexes were determined by agarose gel electrophoresis. These complexes were prepared using varying amounts of PLA/PEI nanoparticles (10-120 μg/mL) and pDNA (1 μg) in PBS and incubating for 20 min at room temperature. Complexes were then electrophoresed through 1% agarose gel at 100 V for 45 min. After electrophoresis, gels were stained with ethidium bromide and visualized on a UV-transilluminator. A DNA ladder (1 kb) (Bioneer, San Diego, CA) was used as a size marker.

Determination of transfection efficiency

Human embryonic kidney (HEK) 293 cells and human cervix epithelial carcinoma (HeLa) cells were used for the transfection experiments. HEK 293 cells were seeded at 2×10^5 cells/well in 12-well plates. After 24 h incubation, *in vitro* transfections were performed as follows. pDNA-loaded nanoparticles were added to each well with fresh serum-free medium, and then incubated in a 5% CO_2 incubator at 37°C. After incubation for 8 h, the medium was replaced with DMEM containing FBS and cells were then incubated in the 5% CO_2 incubator at 37°C for an additional 40 h. The cells were then lysed by adding Promega reporter lysis buffer. β-Galactosidase activity in the cell lysates was determined by measuring absorbance at 420 nm using an ELISA microplate reader (ELX808, BioTEK, U.S.A.).

HeLa cells were seeded at 2×10^5 cells/well in 12-well plates 24 h prior to transfection. HeLa cells were transfected using the same method as for HEK 293 cells. PLA/PEI nanoparticles were complexed with pEGFP-C1 and transfected in HeLa cells. After 48 h transfection, cells were washed twice with PBS, and harvested with trypsin/

EDTA. Cells were collected by centrifugation and washed with PBS. Finally, the transfected cells were analyzed by fluorescence microscopy (BX50, Olympus, Japan) and flow cytometry (Epics XL, Coulter, U.S.A.).

Cell viability

Cytotoxicity evaluations were performed using the MTT assay. Briefly, after transfection experiments, media were replaced by 1 mL fresh medium containing 10% FBS. 50 μL of 2 mg/mL MTT solution in PBS was then added and the plates were incubated for an additional 4 h at 37°C. The MTT-containing medium was then removed and 300 μL dimethylsulfoxide was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader.

Statistical analysis

Transfection and cell viability experiments were performed at least in quadruplicate. Data are presented as mean±standard deviation. Statistical significance and multiple comparison test were performed using the unpaired student's t-test and ANOVA analysis (SPSS version 12.0) determined at the 95% or 99% confidence level.

RESULTS AND DISCUSSION

Size of nanoparticles

PLA/PEI nanoparticles (weight ratio=5:1) were prepared using the described double emulsion-solvent evaporation method, and were of a spherical morphology (data not shown). It was previously found that particle size significantly affected cellular and tissue uptake, and that some cell lines only took up submicron particles (Panyam *et al*., 2003). In the present study, particle size was dependent on the amount of the PVA used as a stabilizing agent, as shown in Table I. Nanoparticle size decreased as the proportion of PVA increased as PVA is highly hydrophilic and thus lowers interfacial energy at the water/polymer interface. These results are consistent with those of an earlier study (Zambaux *et al*., 1998).5% (w/v) PVA was chosen as the optimum amount. Polydispersity indices less than 0.2 indicated that the nanoparticles had a narrow size distribution (Tiyaboonchai *et al*., 2003). There was little difference between the sizes of nanoparticles without pDNA, regardless of the molecular weight of PEI, whereas the size of nanoparticles with pDNA increased as the PEI molecular weight increased.

Table I. PLA/PEI nanoparticle size and its dependence on PEI molecular weight (n=3)

Mw of PEI	pDNA	PVA (wt %)	Size (nm)	Polydispersity index
0.8K	-	5%	206 ± 32	0.20
0.8K	+	5%	230 ± 41	0.13
25K	-	1%	305 ± 36	0.17
25K	-	3%	268 ± 15	0.15
25K	-	5%	255 ± 21	0.19
25K	+	5%	426 ± 81	0.26
25K	-	10%	290 ± 32	0.35
750K	-	5%	233 ± 31	0.21
750K	+	5%	636 ± 90	0.30

* Weight ratio of polymer to pDNA was 50:1.

Complex formation

Complex formation between nanoparticles and pDNA was analyzed by agarose gel electrophoresis, as described in Materials and Methods. Plasmid pEGFP-C1 was mixed with varying amounts of nanoparticles and the resulting complexes were electrophoresed through a 1% agarose gel. The increase in the PLA/PEI to pDNA weight ratio caused the intensity of the pDNA band in the gel to reduce to zero, indicating that all of the pDNA was trapped on the nanoparticle surface (Fig. 1 C and D). In contrast, with the PLA and PLA/PEI 0.8 K nanoparticle system, pDNA migrated in the gel, as shown in Fig. 1 A and B. This indicated that PLA nanoparticles without PEI or with 0.8K PEI could not complex with pDNA. Electrophoresis results suggested that pDNA bound to the imine groups of PEI by electrostatic interaction, and that it was adsorbed at the surface. It is likely that the imine group of PEI bound with pDNA phosphate groups by polyelectrolytic interaction. This hypothesis is consistent with the results of the zeta-potential measurements.

Zeta-potentials of pDNA-loaded nanoparticles

Surface charge is an important determinant of electrostatic interaction, and the surface charge of DNA delivery systems critically affects transfection efficiency (Kim *et al*., 1998). Thus, the zeta-potentials of PLA/PEI and pDNA complexes were determined and the results are summarized in Table II. The zeta-potentials became positive on increasing the weight ratio of PLA/PEI 25K and PLA/PEI 750K to pDNA in nanoparticles, whereas PLA and PLA/PEI 0.8 K nanoparticles remained negative even when the polymer to DNA weight ratio was increased. The negative potentials of PLA nanoparticles were attributed to the terminal carboxyl groups of PLA (Maruyama *et al*., 1997). The PLA/PEI 0.8K system showed negative zeta-potential values throughout the various ranges of the PLA/PEI 0.8 K to pDNA weight ratio examined. In addition, when the concentration of PEI was increased, the zeta-potentials of the resulting nanoparticles were increased, presumably because of an increase in surface density of PEI.

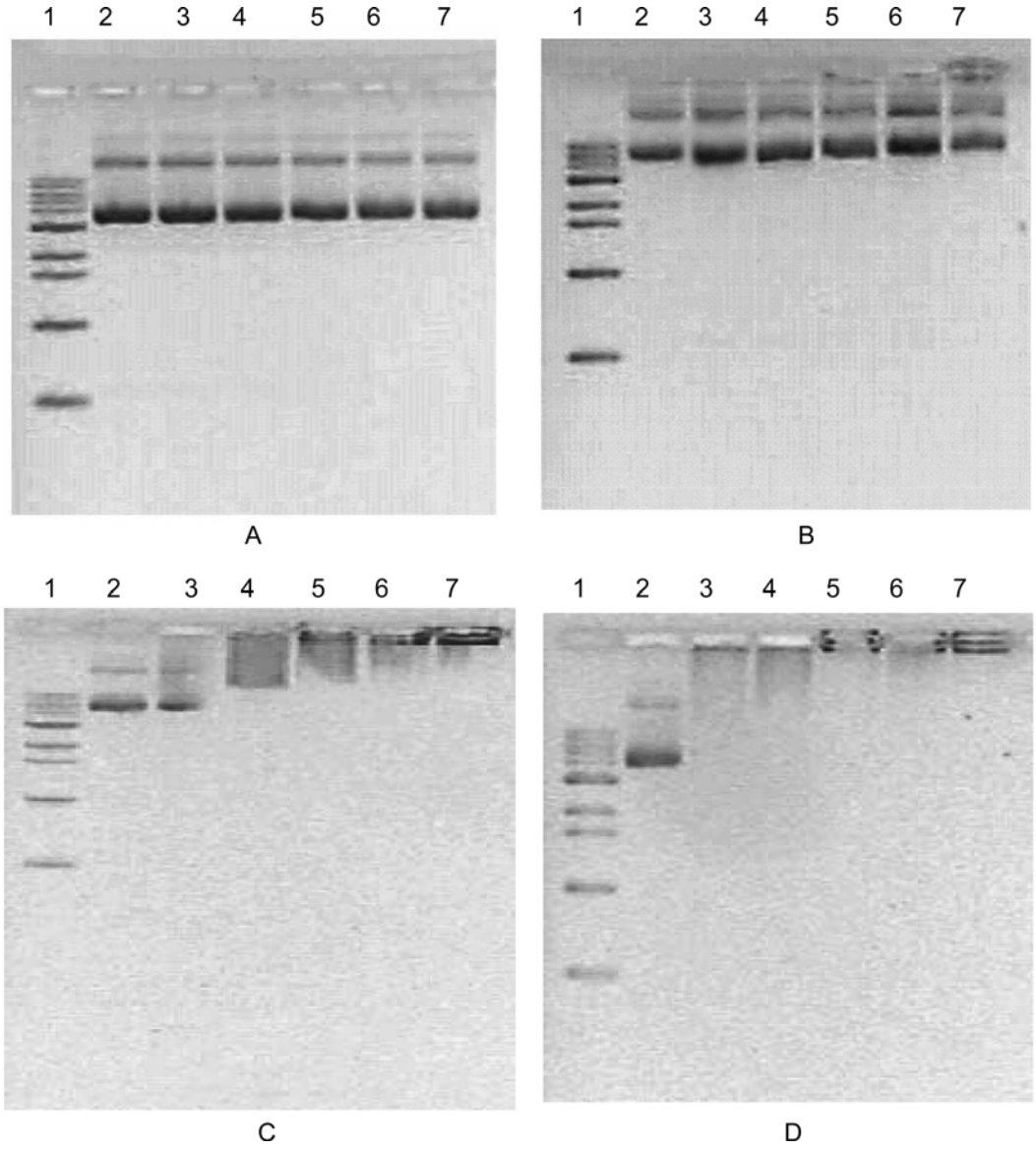

Fig. 1. Gel electrophoresis of nanoparticle/pDNA (pEGFP-C1) complexes on 1% agarose gel. (lane 1) Mw Marker; (lane 2) pEGFP-C1 alone; (lane 3-7) complexes of pEGFP-C1 with increasing levels of nanoparticles (10, 30, 50, 80, or 120 w/w ratios). A; PLA nanoparticle, B; PLA/PEI 0.8K nanoparticle, C; PLA/PEI 25K nanoparticle, D; PLA/PEI 750K nanoparticle.

Table II. Effect of the ratio of PLA/PEI to pDNA (w/w) on the zeta-potential (mV) of pEGFP-C1 complexes (n=3)

Weight ratio	10	30	50	80	120
PLA	-37.4±1.0	-33.7±0.7	-36.9±0.5	-36.9±0.6	-38.5±0.3
PLA/PEI 0.8K	-19.5±3.7	-18.6±1.2	-3.95±1.9	-5.18±2.5	-4.2±1.7
PLA/PEI 25K	-3.2±0.3	0.6±2.5	7.8±2.8	10.7±5.3	16.6±1.2
PLA/PEI 750K	-5.1±2.3	11.0±2.4	12.4±1.7	17.5±2.1	20.4±7.9

Effect of PEI molecular weight on transfection efficiency

Transfection efficiencies were measured by detecting β-galactosidase activity in the HEK 293 cell line. The effect of PEI molecular weight on the transfection of the PLA/PEI nanoparticles complexed with pCMV/β-gal was examined. The commercial transfection reagent, Lipofectin™, was used as a control. The PLA/PEI 25K system was found to have a transfection efficiency comparable to that of Lipofectin™, as shown in Fig. 2. The transfection efficiency of the PLA/PEI 25K system was higher than those of the PLA/PEI 0.8 K and PLA/PEI 750K systems. The low efficiency of the PLA/PEI 0.8 K system might be due to a lack of pDNA binding, which was consistent with the negative zeta-potential. Even though the PLA/PEI 750K system showed positive zeta-potential, this system also

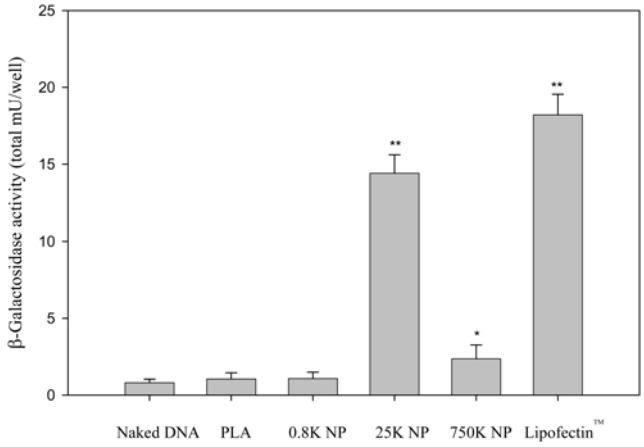

Fig. 2. Transfection efficiencies of various PLA/PEI systems containing PEIs with different molecular weights. Transfection efficiencies were determined by measuring β-galactosidase activity in HEK 293 cell lines (n=3). The weight ratios of polymers and Lipofectin™ to pDNA were 30:1 and 3:1, respectively. Results are presented as mean ± S.D. (*p< 0.05 and **p<0.01 compared with the naked DNA).

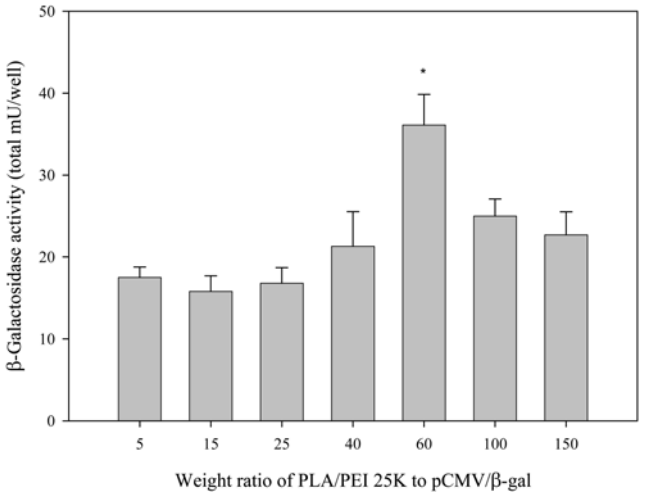

Fig. 3. Effect of the quantity of PLA/PEI 25K nanoparticles on β-galactosidase activity in HEK 293 cell lines (n=4). Experimental conditions were the same as those described in Fig. 2. Results are presented as mean ± S.D. (*p<0.01 compared with other results).

exhibited a lower transfection efficiency than the PLA/PEI 25K system. From the particle size measurements, PLA/PEI 25K/pDNA complexes were smaller than the PLA/PEI 750K/pDNA complexes. It would be expected that the larger size of the PLA/PEI 750K system would inhibit its uptake by the cells.

Effect of the PLA/PEI to pDNA weight ratio on the transfection efficiency

Transfection efficiency and cytotoxicity have been shown to be dependent on the N/P ratios (PEI:DNA) (Godbey *et al*., 1999), and transfection efficiency increased on increasing amount of PLA/PEI 25K nanoparticles in HEK 293 cells (Fig. 3). The highest transfection efficiency was achieved at a 60:1 weight ratio. The transfection efficiency of PLA/PEI 25K nanoparticles above this 60:1 weight ratio reduced with increasing nanoparticle/DNA ratio. Based on the results from the ANOVA and multiple comparison test, the value of transfection efficiency at weight ratio 60 was significantly different with the other remaining results (p< 0.01). This result suggested that an optimal range of nanoparticles seemed to be a ratio of around 60:1.

The results of β-Galactosidase assays performed in HeLa cells were similar to those in HEK 293 cells (data not shown). The transfection efficiency was analyzed with flow cytometry after the transfection of pEGFP-C1 as shown in Fig. 4. The transfection efficiency of PLA/PEI nanoparticles loaded with pEGFP-C1 in HeLa cells was about 40% of that of the Lipofectin™ system (data not shown). Generally, transfection efficiency is known to be dependent on the cell line used (Prabha *et al*., 2002). The transfection efficiency in HeLa cells was also highest at a weight

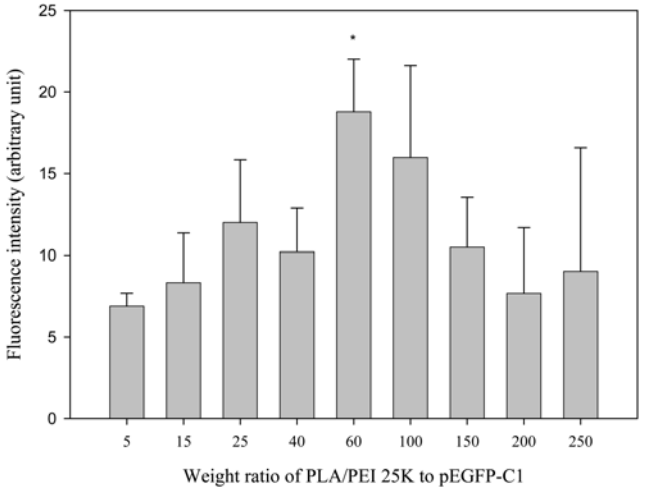

Fig. 4. Transfection efficiency of PLA/PEI 25K nanoparticles/pEGFP-C1 analyzed by flow cytometry in HeLa cells (n=4). The conditions were the same as those for HEK 293 cells. Results are presented as mean ± S.D. (*p<0.05 compared with the results at weight ratio 5, 15 and 200).

ratio of 60:1, as seen in HEK 293 cells, but there was no significance differences except results at weight ratio 5, 15 and 200.

Fig. 5 shows a fluorescence microphotograph of the transfected HeLa cells with PLA/PEI nanoparticles loaded with pEGFP-C1. The transfection efficiency of the PLA/PEI 25K system (60:1 weight ratio) was comparable to that of the commercial Lipofectin™ system.

Cell viability

The cytotoxic effects of PLA and PLA/PEI nanoparticles were estimated 48 h post-transfection using MTT colori-

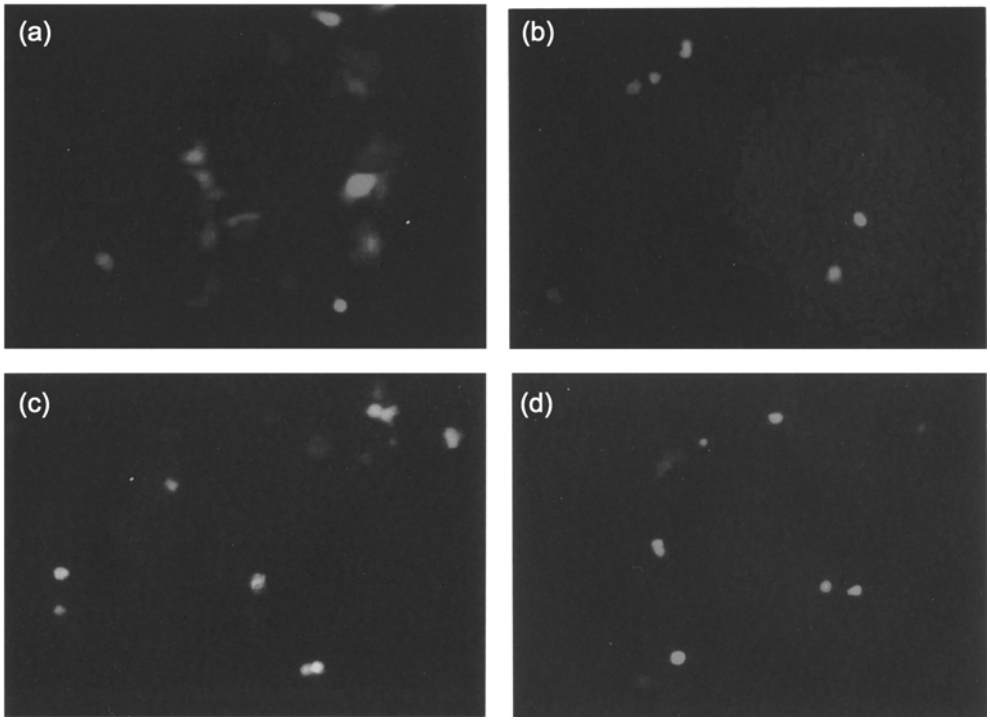

Fig. 5. Fluorescence microphotograph of pEGFP-C1 expression in HeLa cells (×100). The reagents used for transfection were (a) Lipofectin™ 3 μg/g DNA, (b) PLA/PEI 25K 15 μg/g DNA (c) PLA/PEI 25K 60 μg/g DNA and (d) PLA/PEI 25K 150 μg/g DNA.

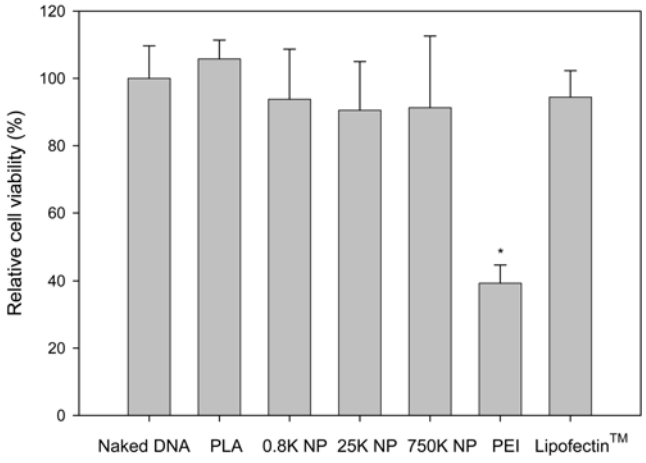

Fig. 6. Relative cell viabilities for various PLA/PEI systems (n=6). HeLa cells were used for this testing. The molecular weights of the PEI in the PLA/PEI nanoparticle system were 0.8K, 25K and 750K. Experimental conditions were the same as those described in Fig. 2. The N/P ratio of PEI to pDNA was 9. Results are presented as mean ± S.D. (*p<0.01 compared with the naked DNA).

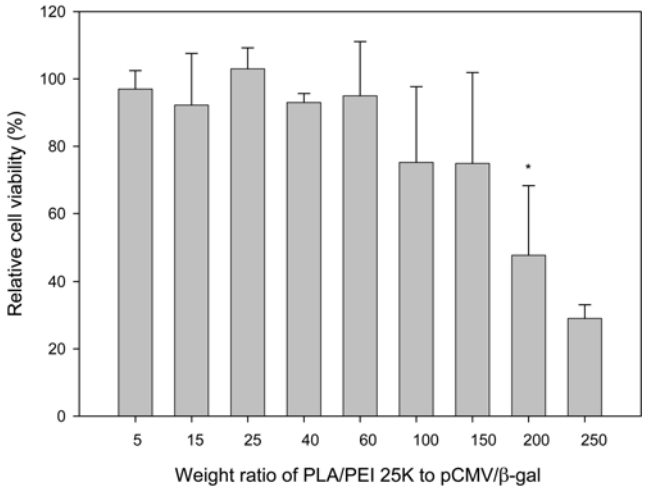

Fig. 7. Effect of the quantity of PLA/PEI 25K nanoparticles on cell viability in HEK 293 cells (n=4). The conditions used were the same as those described for HEK 293 cell transfection. Results are presented as mean ± S.D. (*p<0.05 compared with results at weight ratio less than 60).

metric assays. Average HeLa cell viabilities were >90% after transfection of PLA or PLA/PEI systems, as shown in Fig. 6. A reduction in cytotoxic effect was noted for all PLA/PEI nanoparticle systems. However, PEI was significantly more cytotoxic than the PLA/PEI nanoparticle systems. These results indicate that the PLA/PEI nanoparticle system has a tolerable cell viability. We also tested the relationship between cell viability and PLA/PEI to DNA weight ratio. If tolerable cytotoxicity is defined as a decrease in viable cell levels of no more than 20% compared to control (Gebhart and Kabanov, 2001), PLA/PEI nanoparticles had slight cytotoxic effects with an average cell viability of >90% at a weight ratio of PLA/PEI to pDNA of up to 60:1, as shown in Fig. 7. Based on the results

from the ANOVA and multiple comparison test, result of 200:1 ratio was significantly different with the results at weight ratio less than 60 ($p<0.05$). Above the 200:1 weight ratio, cell viability was reduced as the weight ratio of PLA/PEI to pDNA was increased. This appeared to be due to the cytotoxic effect of PEI which could harm cells by causing lysosomal disruption and fusogenic effects (Godbey *et al*., 1999). Thus, the data indicates that a PLA/PEI to pDNA weight ratio of >200 is unsuitable for nanoparticle production. In summary, PLA/PEI 25K nanoparticles were found to enable satisfactory transfection with good cell viability. We conclude that PLA/PEI nanoparticles might be promising as a pDNA delivery system.

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