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Physicochemical characterization of poly(L-lactic acid) and poly(D,L-lactide-co-glycolide) nanoparticles with polyethylenimine as gene delivery carrier

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Abstract

Polymer nanoparticles have been used as non-viral gene delivery systems and drug delivery systems. In this study, biodegradable poly(L-lactic acid) (PLA)/polyethylenimine (PEI) and poly(D,L-lactide-co-glycolide) (PLGA)/PEI nanoparticles were prepared and characterized as gene delivery systems. The PLA/PEI and PLGA/PEI nanoparticles, which were prepared by a diafiltration method, had spherical shapes and smooth surface characteristics. The size of nanoparticles was controlled by the amount of PEI, which acted as a hydrophilic moiety, which effectively reduced the interfacial energy between the particle surface and the aqueous media. The nanoparticles showed an excellent dispersive stability under storage in a phosphate-buffered saline solution for 12 days. The positive zeta-potentials for the nanoparticles decreased and changed to negative values with increasing plasmid DNA (pDNA) content. Agarose gel electrophoresis showed that the complex formation between the nanoparticles and the pDNA coincided with the zeta-potential results. The results of in vitro transfection and cell viability on HEK 293 cells indicated that the nanoparticles could be used as gene delivery carriers.

Keywords: Poly(L-lactic acid); Poly(D,L-lactide-co-glycolide); Polyethylenimine; Nanoparticles; Gene delivery

1. Introduction

Gene therapy is a promising concept for treating diseases, and a wide variety of vectors to deliver therapeutic genes into the desired target cells have been studied. An ideal gene delivery carrier would be a

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system that can safely transport the genetic materials without exhibiting any toxicity and immune responses, and can be produced on a large scale (Smith et al., 1997). Most studies used the viral method using either a retrovirus and adenovirus or a non-viral method using liposomes. Recently, the use of non-viral vectors such as polycationic polymers (Choi et al., 1998; Kabanov et al., 2002), polymeric micelles (Kakizawa and Kataoka, 2002) and nanoparticles (Perez et al., 2001; Rhaese et al., 2003) to deliver plasmid DNA (pDNA) have been highlighted. Polycationic polymers, particularly polyethylenimine (PEI), have the ability to bind with pDNA easily, and can be applied to a wide variety of cells (Godbey et al., 1999).

Nanoparticles have been used to deliver medicines, and have advantages such as a high stability, easy uptake into the cells by endocytosis, and the targeting ability to specific tissues or organs by adsorption or coating with ligand materials at the surface of the particles (Lobenberg et al., 1997). In particular, biodegradable nanoparticles are available for delivering drugs and degradation at a specific site (Belbella et al., 1996). Poly(lactic acid) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) polymers, which are biocompatible and biodegradable, have been approved by the FDA for certain human clinical uses (Sahoo et al., 2002). The degradation time of PLGA can be altered from days to years by varying the polymer molecular weight, the lactic acid to glycolic acid ratio in copolymer, or the structure of nanoparticles. Biodegradable nanoparticles have been suggested to be good gene delivery carriers because of the possibility of achieving a safe and sustained action (Prabha et al., 2002). There are two types of nanoparticle system carrying DNA, a DNA entrapment system (Leong et al., 1998; Hirosue et al., 2001) and a surface binding system (Ravi-Kumar et al., 2004; Rhaese et al., 2003). DNA entrapment system is the common nanoparticle system, which encapsulates the pDNA. This system has the advantages of pDNA protection along with the controlled release. Surface binding systems utilize the ionic interaction between the cationic polymers and the anionic pDNA.

PEIs are used extensively as non-viral DNA delivery vehicles both in vitro and in vivo. For efficient transfection, PEIs already have the intrinsic characters of DNA condensation and endosomal release (Zheng et al., 2000). Moreover, PEIs have been modified to have additional features such as target specificity and improved biocompatibility for in vivo gene delivery.

This study developed a novel biodegradable polymeric nanoparticles composed of the blends of PLA, PLGA 50:50 (PLGA50), or PLGA 75:25 (PLGA75) with PEI. The PLA/PEI, PLGA50/PEI and PLGA75/PEI nanoparticles are types of surface binding systems for gene delivery, and have the advantages of the ease binding and condensation with pDNA, pDNA protection and the avoidance of the direct contact with organic solvents during the process of nanoparticle preparation. This paper reports the preparation and characterization of PLA/PEI and PLGA/PEI nanoparticles containing pCMV-β-gal as a model DNA. In addition, the effects of the polyethylenimine on the nanoparticles characteristics and DNA adsorption efficiency were also evaluated.

2. Materials and methods

2.1. Materials

Polymers such as PLA (Mw = 10,600), PLGA50 (Mw = 50,000–75,000), PLGA75 (Mw = 90,000–126,000) and PEI (Mw = 64,000) for the preparing nanoparticles were obtained from Sigma (St. Louis, MO, USA). The ethidium bromide, agarose, fetal bovine serum (FBS) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were also purchased from Sigma. The β-galactosidase assay kit was obtained from Qiagen (Valencia, CA, USA). All the solvents were of HPLC grade and the other reagents were of analytical grade.

2.2. Preparation and characterization of nanoparticles

The PLA/PEI and PLGA/PEI nanoparticles were prepared by a diafiltration method (Oh et al., 1999). Either PLA or PLGA (25 mg) dissolved in 10 ml of dimethylsulfoxide was added to a definite amount of a PEI solution (2.5–15 mg) with constant stirring. The solution (15 ml) was dialyzed against distilled water in dialysis tubing (MWCO 12,000 g/mol) pretreated according to the manufacturer's instructions (Sigma).

The medium was replaced every 1 h for the first 3 h and every 3 h for 2 days. The solution was then lyophilised using a freeze dryer and stored in a vacuum desiccator at room temperature. For physicochemical characterization, pDNA-loaded nanoparticles were prepared by mixing the nanoparticles with a pDNA solution (2 μg/ml) at room temperature.

The mean particle size and size distribution of nanoparticles were measured before and after lyophilization using a dynamic light scattering particle size analyzer (Mastersizer E, Malvern, UK). The size and shape of nanoparticles were observed by scanning electron microscope (SEM) (JSM5400, JEOL, Japan). The zeta-potential of nanoparticles in a phosphate-buffered saline (PBS) solution (pH 7.4) was determined using a Malvern Zetasizer 3000 E (Malvern, UK).

2.3. DNA adsorption on nanoparticles and dispersive stability of nanoparticles

The adsorption quantity of pDNA on the nanoparticle surface was analyzed as a function of the pDNA concentration (0.2–15 μg/ml). A fixed amount of nanoparticles was incubated with various pDNA solutions in 1 ml of PBS at 25 °C for 1 h, and the suspension was centrifuged at 25,600 rpm for 10 min. The absorbance of the supernatant was measured at 260 nm using a UV spectrophotometer (Hewlett Packard, USA). The dispersive stability of nanoparticles in suspension was evaluated using a turbidimeter (2100N, Hach, USA). The nanoparticles containing various PEI concentrations were dispersed in water (200 μg/ml) by sonication for 10 min and incubated at room temperature for 12 days with constant checking the turbidity at the indicated time points.

2.4. Agarose gel electrophoresis of pDNA and nanoparticle complexes

Complex formation between nanoparticles and pDNA was analyzed by 1% agarose gel electrophoresis. The gels were prepared with 1% (w/v) agarose in Tris-acetate–EDTA buffer (pH 8.3). A fixed amount (1 μg) of pDNA was complexed with various amounts of nanoparticles in 20 μl of PBS. Gel electrophoresis was performed at a constant 100 V for 1 h. The gel was stained with ethidium bromide (0.5 μg/ml) and photographed on a UV trans-illuminator to show the location of the pDNA. Lambda DNA ladders (Bioneer, San Diego, CA, USA) were used as a DNA size marker.

2.5. In vitro transfection and cell viability

Human embryonic kidney (HEK) 293 cells were seeded 24 h prior to transfection into 12-well plates at a density of 2×10^5 cells per well in 1 ml of complete medium. The DNA-loaded nanoparticles were prepared by mixing pDNA (2 μg) with nanoparticles (50 μg) in a serum-free medium and incubating them for 30 min at room temperature. The cells were washed once with serum-free medium, and the complex solution was added by dropping. After incubation for 4 h at 37 °C in a 5% CO_2 incubator, the medium was replaced with fresh medium containing 10% FBS. After transfection for 48 h, the β-galactosidase activity was determined using a β-galactosidase enzyme assay system with the reporter lysis buffer (Promega, Madison, WI, USA). Briefly, the growth medium was removed from the cells in 12-well plates and the cells were washed carefully with PBS twice in order to prevent the detachment of cells. The cells were lysed with the reporter lysis buffer by incubating them for 30 min on a rocker. The cells were then collected into a microtube and centrifuged at 17,000 rpm for 5 min at 4 °C. Fifty microliters of the supernatant was transferred to a 96-well plate, and a 50 μl of assay buffer was added. The solution was incubated at 37 °C for 30 min. The reaction was quenched by adding 150 μl of 1 M sodium carbonate, and the absorbance at 420 nm was read using a microplate reader (ELX808, Bio-Tek, USA) for the β-galactosidase activity.

The cytotoxicity of nanoparticles was evaluated by MTT assay. At the end of the transfection experiment, the media was replaced by 1 ml of fresh medium containing 10% FBS. Fifty microliters of a 2 mg/ml MTT solution in PBS was added and the plates were incubated for an additional 4 h at 37 °C in a 5% CO_2 incubator. The MTT-containing medium was removed and the formazan crystals formed by the living cells were dissolved in 300 μl of dimethylsulfoxide. The absorbance was measured at 570 nm using a microplate reader after 1 h incubation. The cell viability (%) was calculated as the absorbance ratio of samples compared with the untreated control.

3. Results

3.1. Particle size of nanoparticles

The PLA/PEI or PLGA/PEI nanoparticles were prepared by a diafiltration method and the yields of nanoparticles were in excess of 93%. The particle size significantly affects the level of cellular and tissue uptake, and in some cell lines, only submicron size particles are efficiently taken up (Panyam and Labhasetwar, 2003). In this respect, the size and size distribution of nanoparticles is important and should be characterized carefully. As shown in Table 1, the size of nanoparticles depended on the amounts of PEI. The nanoparticles had a narrow sized distribution with polydispersity index lower than 0.3. The size of nanoparticles decreased as the amount of PEI in the nanoparticles was increased. The nanoparticles without PEI aggregated after lyophilization. There was little disparity in the size and size distribution of nanoparticles containing PEI before and after lyophlilization (data not shown). However, the aggregated particles after lyophilization were re-dispersed easily by slight stirring or sonication. The hydrophilic polymer decreased the interfacial energy at the water/PLA interface more effectively (Maruyama et al., 1997). Therefore, the hydrophilic PEI might have affected the formation of nanoparticles in the case of PLA/PEI and PLGA/PEI. The decrease in particle size suggested that the positive zeta-potential characteristics prevented the particles from approaching each other during solidification of nanoparticles. The size of nanoparticles was similar, regardless of the types of polymers used. The morphology of nanoparticles observed by SEM was almost spherical, smooth and non-porous, and the surface was homogeneous in the nano-sized range, as shown in Fig. 1.

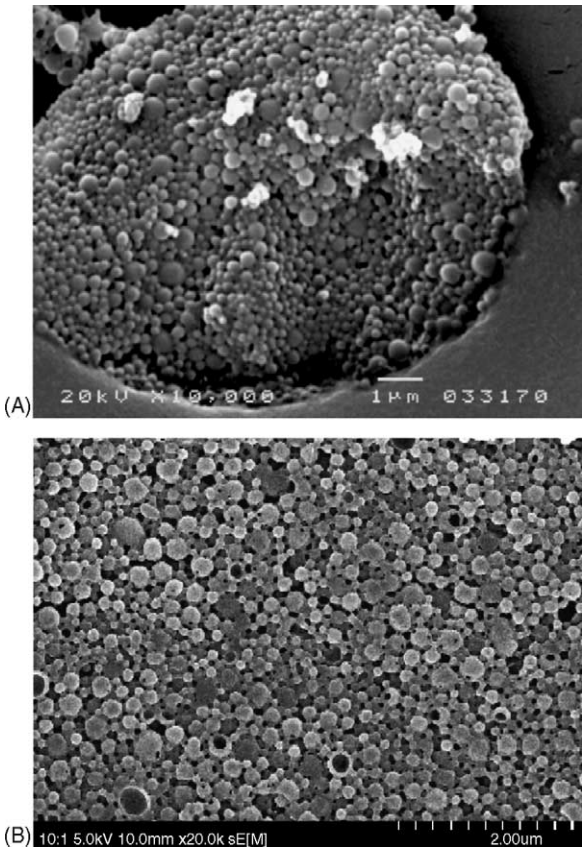

Fig. 1. Scanning electron microphotographs of nanoparticles. (A) PLA nanoparticle and (B) PLA/PEI (5:1) nanoparticle.

3.2. Surface potential of nanoparticles

The surface charge of the DNA delivery system is one of the critical factors affecting the transfection efficiency (Kim et al., 1998). The zeta-potential of

Table 1
Average particle size of nanoparticles prepared by the diafiltration method ($n = 3$)

Weight ratio[a]	PLA/PEI		PLGA50/PEI		PLGA75/PEI	
	Particle size (nm)	Polydispersity index	Particle size (nm)	Polydispersity index	Particle size (nm)	Polydispersity index
0	560 ± 55	0.28	581 ± 62	0.29	625 ± 67	0.32
0.1	462 ± 44	0.27	363 ± 35	0.25	391 ± 38	0.26
0.2	320 ± 30	0.20	294 ± 28	0.22	283 ± 27	0.22
0.3	307 ± 27	0.19	258 ± 23	0.18	262 ± 22	0.20
0.4	263 ± 21	0.18	207 ± 18	0.18	214 ± 19	0.18
0.6	217 ± 15	0.18	186 ± 12	0.17	177 ± 13	0.17

[a] Weight ratio of PEI to PLA or PEI to PLGA during preparation of nanoparticles.

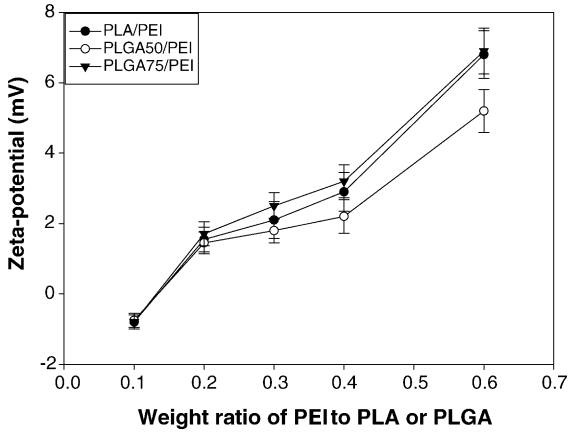

Fig. 2. Effect of PEI on the zeta-potential of nanoparticles with 1 μg of pDNA at 25 °C in PBS ($n = 3$).

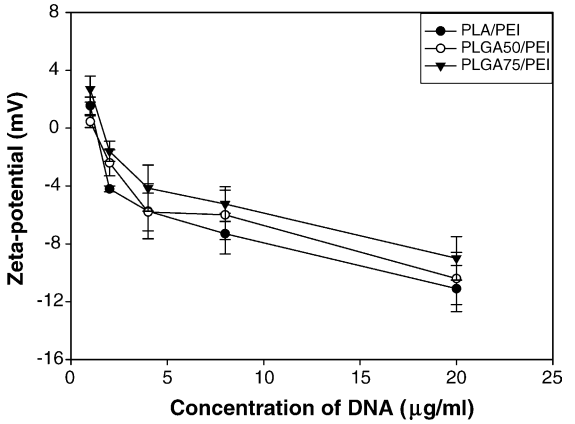

Fig. 3. Zeta-potential of nanoparticles complexed with pDNA in PBS ($n = 3$). Weight ratio of PLA or PLGA to PEI was 5:1.

PLA/PEI and PLGA/PEI nanoparticles was determined based on the quantity of PEI. As shown in Fig. 2, the zeta-potential of nanoparticles containing PEI showed a highly positive zeta-potential in PBS. In contrast, the zeta-potential of nanoparticles without PEI showed a negative zeta-potential. The negative value of the PLA nanoparticles was due to the terminal carboxyl groups of the PLA and the hydrophobic characteristic of the surface (Maruyama et al., 1997). This indicated that the PEI moiety was stably associated with the surface of nanoparticles. In addition, the zeta-potential of the resulting nanoparticles increased when the PEI concentration was increased. This suggests an increase in PEI in the vicinity of the surface of nanoparticles.

As shown in Fig. 3, the zeta-potential of nanoparticles was changed from positive to negative value because of the negative zeta-potential of DNA. As the DNA was added to the nanoparticles suspension, the sign of the potential changed to a negative value. This suggests that the phosphate group of DNA was adhered to the PEI of nanoparticles surface through electrostatic interactions.

3.3. Gel retardation assay and adsorption of pDNA onto the surface of nanoparticle

From the gel retardation assay, the complexation of pDNA with the nanoparticles was determined. The plasmid DNA mixed with various amounts of nanoparticles was resolved by electrophoresis on 1% agarose gel. As shown in Fig. 4, an increase in the amount of nanoparticles caused the gradual disappearance of

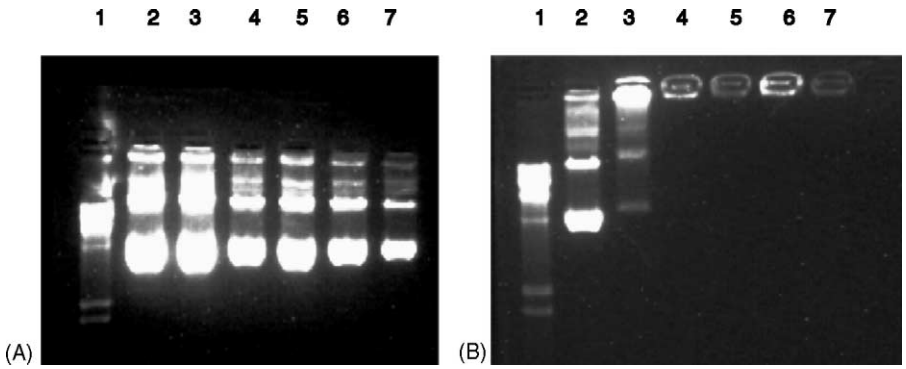

Fig. 4. Gel electrophoresis of the pDNA and nanoparticles. (A) PLA nanoparticle and (B) PLA/PEI (5:1) nanoparticle. (1) Marker; (2) pDNA; (3–7) nanoparticle complexed with pDNA (the amounts of the PLA/PEI nanoparticles) were 10, 30, 50, 80 and 120 μg, respectively.

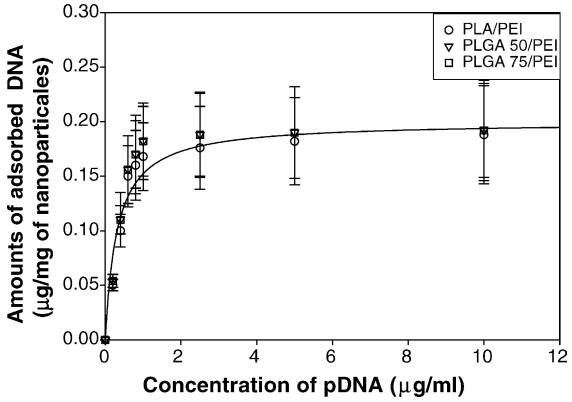

Fig. 5. Adsorbed amount of pDNA on the nanoparticle as a function of the DNA concentration at 37 °C ($n = 3$). The PLA to PEI or PLGA to PEI weight ratio was 5:1.

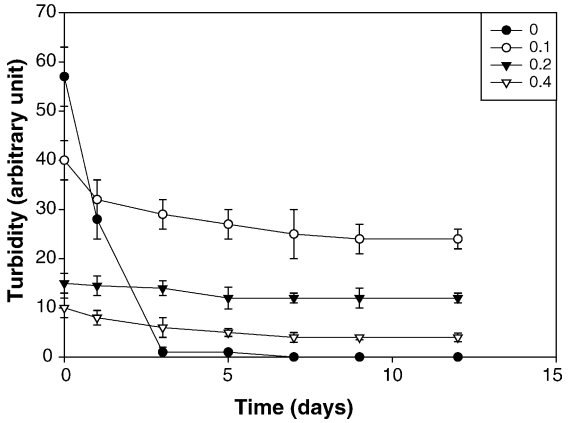

Fig. 6. Dispersive stability of the PLGA75/PEI nanoparticles depending on the PEI to PLGA weight ratio ($n = 3$).

pDNA. At a nanoparticles to pDNA weight ratio > 10:1, the pDNA did not migrate at all, and the result showed that all the pDNA was trapped on the surface of nanoparticles. In the case of nanoparticles without PEI, a spot of pDNA remained despite the increase in the quantity of nanoparticles as shown in Fig. 4(A). This suggests that pDNA can bind to PEI by an electrostatic interaction and be adsorbed at the surface of nanoparticles. This result coincided with the zeta-potential results at the surface of nanoparticles.

The adsorption of pDNA on the surface of the various nanoparticles largely depended on the quantity of PEI, as illustrated from the Fig. 5. The adsorption of pDNA reached a plateau as the concentration of pDNA was increased. The adsorption or adsorption pattern was similar irrespective of the type of polymers.

3.4. Dispersion stability of nanoparticles

Nanoparticles should have dispersive stability in an aqueous media and during storage. The dispersive stability of nanoparticles was analyzed from the change in turbidity of various nanoparticle suspensions. The suspension of nanoparticles was tested in the cases of nanoparticles with and without PEI. The suspensions were stored at room temperature for 12 days. As shown in Fig. 6, the nanoparticle suspension without PEI indicated the aggregate formation as illustrated by the rapid decrease in turbidity in only 3 days. In contrast, the turbidity of nanoparticles with PEI was almost constant for 12 days except for the case of 10% PEI addition. Therefore, the result suggested that the imine portion of the PEI on the nanoparticles surface was effective in preventing the aggregation of nanoparticles. The PLA/PEI and PLGA/PEI nanoparticles were surrounded by the imine moiety of the PEI protruding into the continuous aqueous phase. This hydrophilic moiety on the nanoparticle surfaces prevented particle aggregation due to the so-called “exclusion effect” (Maruyama et al., 1997).

3.5. In vitro transfection and cell viability

The transfection efficiency on HEK 293 cells was determined by measuring the β-galactosidase activity using a β-galactosidase enzyme assay system, as describing in Section 2. Nanoparticles of PLA/PEI or PLGA/PEI containing the pDNA were introduced into the cell followed by the expression of β-galactosidase. The transfection activity of nanoparticles with PEI was approximately 50% of PEI alone (Fig. 7). In the case of the PLA nanoparticles without PEI, very low β-galactosidase activity was observed and the activity was similar to the control group, where naked pDNA was used. This was because the PLA nanoparticles without PEI could not form a complex with the pDNA.

The cytotoxicity of the PLA/PEI and PLGA/PEI nanoparticles was estimated by MTT assay. The average cell viability of nanoparticles was in excess of 90%, as shown in Fig. 8. From these results, the PLA/PEI or PLGA/PEI nanoparticles appeared to be safer than the PEI alone showing an average cell viability of around 90%.

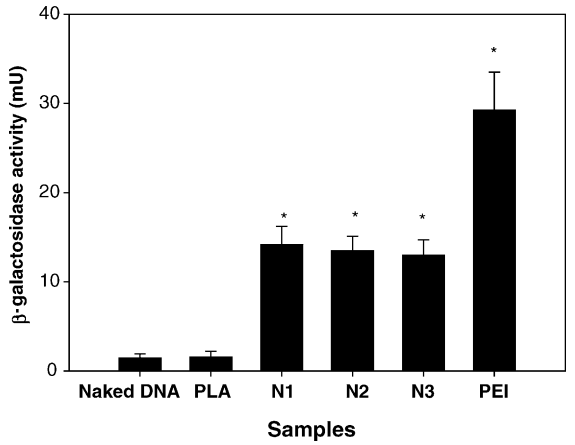

Fig. 7. Transfection efficiency of nanoparticles in the HEK 293 cell line ($n = 6$). N1, N2 and N3 represent the PLA/PEI (5:1), PLGA50/PEI (5:1) and PLGA75/PEI (5:1) nanoparticles complexed with 2 μg of pDNA, respectively. The cells were seeded at 2×10^5 cells/well. The results are shown as a mean ± S.D. ($^{*}p < 0.01$ compared with the naked DNA).

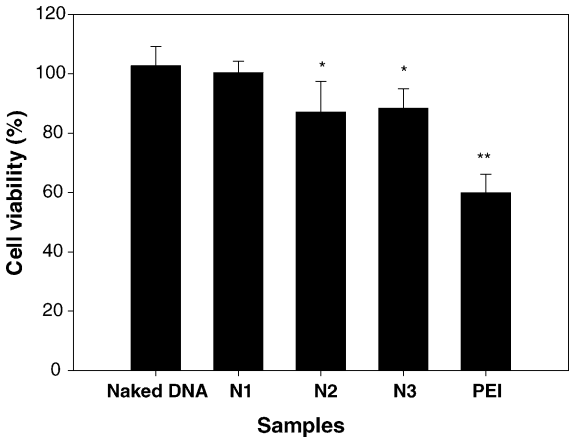

Fig. 8. Cell viability of nanoparticles in the HEK 293 cell line ($n = 6$). N1, N2 and N3 represent the PLA/PEI (5:1), PLGA50/PEI (5:1) and PLGA75/PEI (5:1) nanoparticles complexed with 2 μg of pDNA, respectively. The results are presented as a mean ± S.D. ($^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the naked DNA).

4. Discussion

The advantages of nanoparticles as a drug carrier and the complexation ability with pDNA need to be combined in order to use nanoparticles as a gene delivery carrier. Therefore, cationic polymers are required to maximize the binding with pDNA. The preparation of nanoparticles under optimum conditions is important in terms of the physicochemical properties of nanoparticles.

The diafiltration method has a merit for a nanoparticle preparation without surfactants. PEI has a good affinity with water and has an ability to lower the interfacial energy at the established water/polymer interface, which is the cause of the size reduction. This is similar to the effect of poy(vinyl alcohol) during the preparation of nanoparticles (Murakami et al., 1997).

The dispersion stability of nanoparticles is an important factor in relation to the long storage of a dispersion system. The stability of nanoparticles containing PEI was maintained by electrostatic repulsion of the interparticles because of the positive zeta-potential. The uncharged particles agglomerated when the zeta-potential was reduced to zero (Hoffmann et al., 1997).

PEI has a remarkable ability to transfect a wide range of cells regardless of the size of the genetic material (Godbey et al., 1999). A positive surface charge was one of the properties required to interact with the pDNA. The imine group of the PEI was bound with the phosphate group of pDNA by a polyelectrolytic interaction. The zeta-potential of the nanoparticle surface was a significant factor in determining the electrostatic interaction. The N/P ratio was one of the parameters depending on the transfection efficiency and cytotoxicity (Godbey et al., 1999). In this regard, the determination of the PEI quantity is essential to optimize the pDNA carrier.

The zeta-potential of the nanoparticle surface is the required parameter to determine the optimum condition of complexation. It was reported that the effective zeta-potential value was almost zero (Nguyen et al., 2000). In this respect, a nanoparticle with a low zeta-potential value (PLA or PLGA:PEI = 5:1) was the ideal condition in which all the pDNA was bound at the surface of the particles. The binding of pDNA with the nanoparticles showed a similar physical adsorption, the Langmuir adsorption type, and the associated interparticular force was ionic attraction during the attraction process (Maruyama et al., 1997).

In conclusion, PEI could be introduced to nanoparticles and the PEI content could used to control the size of nanoparticles. PLA/PEI and PLGA/PEI nanoparticles had a high pDNA adsorption capacity and a high dispersive stability. Therefore, the PLA and PLGA

nanoparticles with PEI might be useful in the delivery of pDNA. Future studies will focus on the optimizing the PLA/PEI and PLGA/PEI nanoparticles for in vivo uses as a gene delivery system.

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