Investigation on Characterization and Transfection of a Novel Multi-Polyplex Gene Delivery System

Yu Nie, Wen-Min Yuan, Tao Gong, Jiao Lu, Yao Fu, Zhi-Rong Zhang

Key Laboratory of Drug Targeting, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu 610041, Sichuan Province, China

Received 11 September 2006; accepted 24 April 2007
DOI 10.1002/app.26773
Published online 5 July 2007 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: pDNA was condensed by polycationic peptide polylysine (PLL) to form a core, and then encapsulated in biodegradable monomethoxy (poly ethylene glycol)-poly(lactide-*co*-glycolide)-monomethoxy (poly ethylene glycol) (PELGE) to form core-shell nanoparticles (NPs) as a novel multi-polyplex gene delivery system—PPD(PELGE-PLL-DNA). NPs were prepared by a double emulsification-solvent evaporation technique, using F68 (Pluronic F68, namely Poloxamer 188) as surfactant (not traditional stabilizer PVA), and characterized by morphology, particle size, zeta potential, nuclease, and sonication protection ability, as well as transfection efficiency. Results showed that PPD had a regular spherical shape, with an average diameter of 155 ± 2.97 nm and a zeta potential of −25.6 ± 1.35 mV. PPD could protect plasmid DNA from nuclease degradation and sonication during preparation, while the transfection efficiencies in HepG2 cells and Hela cells were much higher than that of NPs without PLL. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 1028–1033, 2007

Key words: nanoparticle; drug-delivery systems; biocompatibility; gene; transfection

INTRODUCTION

It has been shown that plasmid DNA (pDNA) has great potential in gene replacement therapy, therapeutic application, and vaccines.[1] However, the use of pDNA-based pharmaceuticals as drug substances will largely depend on the development of safe and efficient delivery systems.

Nonviral delivery systems, including cationic liposomes,[2] polypeptides, hydrogelemulsion, and peptide nucleic acid and NPs,[3] are attractive because they are associated with fewer safety concerns and are easy to produce.

Among those systems currently being investigated, biodegradable polymeric NPs with entrapped pDNA have shown the potential for achieving sustained gene expression. NPs are colloidal particles in the nanometer size range and contain a pDNA of interest entrapped in their polymer matrix.[3] Although matrix-type NPs have been formulated using different polymers,[4] such as chitosan, gelatin, and cyclodextrin, NPs formulated from poly(D,L-lactide-*co*-glycolide) (PLGA) and polylactide (PLA) are especially of interest for gene delivery due to their sustained release properties, their ability to protect pDNA from degradation,[5] and its FDA approved biocompatibility and biodegradability.[6]

Recently, we have synthesized PELGE polymer as a novel NPs material, which is based on the PLGA modified with PEG. The newly developed biomaterial was demonstrated by its good blood compatibility[7] according to the International Standard Organization (ISO) and US Pharmacopoeia XXIII recommendations. And then it was taken as a gene carrier,[8] but simple matrix-type NPs had low transfection.[8]

In 1997, Huang and coworkers[9] introduced cationic polypeptide protamine into liposome to form lipid-polycation-DNA lipopolyplexes (LPD), which have appeared promising as efficient gene-delivery vehicles for systemic administration.[10] Therefore, according to its constructional mechanism, the combined use of polycationic peptide and biodegradable macromolecular polymer to be a novel multi-polyplex gene delivery system is presented. PLGA microspheres containing PLL-pDNA were formulated by Capan et al.[11] and PLGA-grafted PLL (PLL-*g*-PLGA) micelles were produced by Jeong.[12] In a previous study, our research group that studied PELGE-NPs loaded with polylysine (PLL) demonstrated that it had rapid escape from the endo-lysosomal compartment into cytoplasm, suggesting the suitability of NPs as a gene delivery vector.[13] It was hypothesized that the introduction of polycationic peptide might better protect pDNA and thus enhances the transfection.

Correspondence to: Z.-R. Zhang (zrzzl@vip.sina.com).
Contract grant sponsor: National High Research and Development Program of China (863 Program); contract grant number: 2004AA27-133142.

In this study, we investigate the integrity of DNA during preparation, the antisonification and DNase properties, and transfection on HepG2 and Hela cells of this system to confirm this thought.

What more is, PVA was used as a stabilizer almost for the preparation of PLGA relative NPs.[3] But this excipient has not approved by FDA for intravascular use. For the systematic use of NPs, suitable surfactant needs to be used. In this study, PLL-pDNA complex-loaded PELGE NPs were prepared by F68 solution as outer water phase, which is authorized for intravascular use, to investigate the transfection efficiency to hepatoma cells and ovarian carcinomas cells of this novel delivery system.

MATERIALS AND METHODS

Materials

PELGE was prepared as described in the literature[8] (M_w = 10,000, determined by GPC). F68 was purchased from Nanjing Weier Company (injection grade), while polyvinyl alcohol (88% hydrolyzed, M_w = 22,000) was from Acros Organics (USA). Poly(L-lysine)-hydrobromide (M_w = 25,000), galactosidase reporter gene staining kit was obtained from Sigma (St. Louis, MO). Plasmid pORF lacZ (3.54 kb) and Lipofectamine™ 2000 was from Invivogen (USA). Plasmid PGL3 and luciferase assay system was from Promega. Hepatoma cell line HepG2 and ovarian carcinomas cell line Hela was obtained from Shanghai Cell Institute, China Academy of Sciences. Cell culture media DMEM and RPMI 1640 were obtained from Gibco (USA). Qiagen Giga Endo-free plasmid purification kit was purchased from Qiagen (CA). Goldview™ DNA dye was obtained from Beijing SBS Genetech. All the other chemicals and reagents used were of the analytical grade obtained commercially.

Preparation of nanoparticles

PELGE-NPs were prepared by a traditional double emulsification-solvent evaporation technique[13] with slight modification of outer water phase and oil phase.

In brief, PLL-pDNA complex (PD, pDNA:PLL ratio = 1 : 1, w/w) was prepared by gentle mixing of 50 μL of pDNA (300 μg/mL) in glucose injection with 50 μL of PLL(300 μg/mL) in the same solution. The mixed solution was subsequently incubated at room temperature for 30 min. PD was emulsified into a solution of 10-mg PELGE in dichloromethylene (DCM) or mixed organic solvent (acetic acid ethyl ester: acetone = 9 : 1(v/v)) by probe sonication for 20 s. Then, 2-mL polyvinyl alcohol (0.5% PVA) or F68 glucose injection solution (1%, w/v) was added to this emulsion. The w/o/w emulsion was obtained following another 20-s sonication and then mixed with 8 mL of the same PVA or F68 solution, followed by reduced pressure rotator evaporation at room temperature to allow solidification of the nanodroplets and elimination of the organic solvents. NPs formed in PVA proceeded extra rinsed with PBS to remove PVA, and finally both kinds of NPs were lyophilized.

Particle size, morphology, and zeta potential

Size of NPs prepared in PVA or F68 solution were measured by both photon correlation spectroscopy (PCS) (Zetasizer Nano ZS90, Malvern instruments, UK) and transmission electron microscope (TEM). One milligram of NPs was diluted in 2 mL of 10 m*M* Hepes buffer and added into the sample cell. The measurement time was set at 2 min (rapid measurement) and each run consisted of 10 subruns. The size distribution follows a lognormal distribution.

For TEM inspection, negative staining electron micrographs of NPs were taken using JEM-100SX electron microscope.

Surface charges of PELGE NPs suspended in Hepes buffer pH 7.4 were determined by zeta potential measurement on a Malvern Zetasizer Nano ZS90 with a mode described earlier. Assays were carried out in triplicate at appropriate sample concentrations.

Integrity of DNA during preparation

pDNA was extracted from PELGE NPs by a chloroform–water extraction method.[14] Briefly, 10 mg of PELGE NPs was dissolved in 1.0 mL of chloroform. To this solution, 1.0 mL of water was added and stirred for 20 min. The two phases were separated by centrifugation for 10 min. The aqueous layer was precipitated by the addition of ethanol, resuspended in 20-μL TE buffer (10 m*M* Tris–HCl, pH8.0), treated with restriction enzymes, and subjected to agarose (1%) gel electrophoresis to determine pDNA integrity. Prior to electrophoresis, PD complex were dissociated by the addition of heparin,[13] to release pDNA from the complex. pDNA combined with loading buffer was then loaded onto the gel and electrophoresed for 1.5 h at 90 V. DNA bands were visualized under UV light after Goldview™ staining.

Stability in DNase I

Naked DNA, DNA loaded PLGA-NPs, PD, and PPD were incubated with DNase I solution (74 U/mL DNA, 10 m*M* Tris–Cl, 150 m*M* NaCl, 1 m*M* MgCl, and pH 7.4) at 37°C for 5 min, 40 min and 1 h, respectively. The enzymatic reaction was stopped by adding of 0.5*M* EDTA.[15] Centrifugation was used for

the collection of NPs, and DMC was used to dissolve the NPs. In addition, 0.9% w/v heparin was added to release DNA from PLL/DNA complexes.[13] The samples were carefully added to the wells of a 0.8% agarose gel (representing 0.5 μg of DNA per well). The gel was run in TBE buffer containing 0.5 μg/mL Goldview™ DNA dye at 100 V for 30 min. Subsequently, the gel was removed from the tank and visualized under UV light by molecular analyst software.

Cell transfection

Hela cells and HepG2 cells were cultured in DMEM and RPMI-1640, respectively, with 10% fetal bovine serum and streptomycin (100 μg/mL). The cells were seeded at 1×10^5 cells per well onto 12-well plates 24-h before transfection. The cells were about 80% confluence at the time of transfection. Then, the cells were washed twice by PBS, and 1 mL of serum-free and antibiotics-free medium was added into each well. For each well in a transfection, NPs-containing 2.5 μg pORF-1acZ and pGL3 plasmid (condensed by PLL or not) were overlaid and mixed gently. The cells were incubated with NPs for 8, 16, and 20 h at 37°C in a CO_2 incubator. Following incubation, NPs was removed and the cell surfaces were rinsed thoroughly and treated with 2 mL of fresh complete medium. Then, the cells were returned to the incubator for another 48 h to allow intracellular gene expression to proceed. Lipofectamine™ 2000 was used as positive control.

X-gal staining

Estimation of the transfection efficiency was performed using β-galactosidase assay. After the desired time of incubation, the cells were washed with PBS twice and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 min at room temperature. Subsequently, the cells were rinsed twice and stained by X-gal (20 mg/mL) according to the manufacture's instructions. The cells were incubated at 37°C overnight and observed under a microscope. The transfected cells were blue after X-gal staining. For each well, five visual fields were chosen randomly. Cells stained blue were counted and the transfection efficiency was calculated as the percentage of the blue cells in each field.

Luciferase assay

pDNA (pGL3) was isolated and purified from DH5-α *Escherichia coli* using the Qiagen Giga Endo-free plasmid purification kit (Qiagen, CA). Luciferase gene expression was determined 48 h after transfection by using a commercial luciferase assay kit (Promega, USA. The luciferase activity was monitored in an Lmax II 384 luminometer (Molecular Devices, USA). The transfection efficiency was expressed as relative light unit per mg of cell protein, the concentration of which was measured by using BCA Protein Assay Kit (Pierce, USA).

RESULTS AND DISCUSSION

Morphology, particle size, and zeta potential

TEM (Fig. 1) demonstrated the similar regular spherical surface of PPD prepared in PVA solution [Fig. 1(a)] and F68 solution [Fig. 1(b)], with similar size about 150–170 nm. Table I shows the average diameter being 246 ± 2.90 nm for PVA while 155 ± 2.97 nm for F68, with a very narrow distribution (polyindex 0.12 ± 0.013 and 0.105 ± 0.095, respectively), determined by Malvern Instruments. Table I illustrates the zeta potential of PPD being −25.6 ± 1.35 mV in 10 m*M* herpes buffer (pH 7.4).

The discrepancy in the size of the same NPs [Figs. 1(a) and 2(a)] is due to the fact that the dynamic light scattering method gives the hydrodynamic diameter rather than the actual diameter of NPs. The particle size is further validated by the TEM of the NPs. In fact, the mean NP diameter measured using TEM is significantly smaller than that obtained with the dynamic light scattering method.

PVA is a commonly used emulsifier in the formulation of NPs, mainly because the NPs formed are smaller and uniform in size and are easy to redisperse in buffer or saline.[16] It has been shown in the previous studies[17] that a fraction of PVA remains associated with the NP surface even after multiple washings. This occurs because the hydrophobic portion of PVA anchors into the NP matrix during their formulation, could not be washed away, and therefore forms the NP interface.[16] This association could

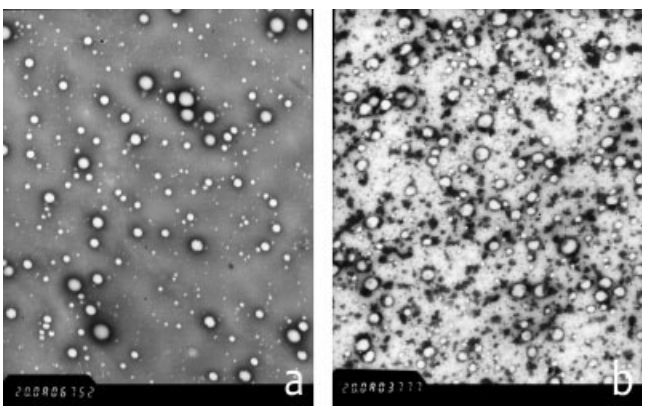

Figure 1 Electronic transmission microscopy comparison of PPD with PVA solution (a) as outer water phase and with F68 solution (b) as outer water phase (×20,000).

TABLE I
Size and Zeta Potential of the PELGE Nanoparticles Formulated Using PVA and F68 Solution (n = 3)

Outer Water Solution	Particle Size (nm)	Polydispersity Index	Zeta potential (mV)
PVA (0.5%)	246 ± 2.90	0.12 ± 0.013	−26.8 ± 2.58
F68 (1%)	155 ± 2.97	0.105 ± 0.095	−25.6 ± 1.35

Data represented as mean ± SD.

contribute toward the hydrodynamic diameter of NPs, which make the detection result be larger than actual size. Furthermore, it was demonstrated that the residual surface-associated PVA affects the physical properties of NPs as well as their cellular uptake.[18]

Pluronic F68 was chosen as nonionic surfactant in preference to ionic surfactants to get nonionic NPs.[19] Pluronic F68, also known as poloxamer 188, is a triblock copolymer PEO–*block*–PPO–*block*–PEO, with a molecular weight of 8400 g/mol and contains around 80% EO w/w. These nonionic surfactants are commonly used to stabilize emulsions and display quite interesting biological properties. Their applications in drug delivery are well-documented.[20] Meanwhile, only a limited number of emulsifiers is commonly regarded as safe to use for parenteral administration, of which the most important is Pluronic F68® (Poloxamer 188).

In our previous study,[13] the introduction of PEG into PLGA-NPs reduces the amount of PVA (from 2 to 0.5%) during preparation. Moreover, in this study, with the change of the oil phase (from DCM to acetic acid ethyl ester:acetone = 9 : 1), F68 was used to substitute PVA as surfactant, which go forward to actual use much.

Integrity of DNA during preparation

Ultrasonic radiation can convert supercoiled DNA to linear and open circular forms, during preparation of NPs, resulting in significant reduction in gene expression. To better distinguish the relative differences in pDNA, status following the formulation of NPs loaded with naked pDNA and PD were analyzed by gel electrophoresis (Fig. 2). Both of them were challenged with 40 W probe sonication in primary emulsion and 80 W in multiemulsion forming. DNA that extracted from NPs showed bands corresponding to that of the supercoiled and circular forms of DNA. As a result, naked DNA and pDNA loaded NPs resulted in complete fragmentation (Fig. 2, lanes 1 and 2), while complexed pDNA remained light supercoiled lane (Fig. 2, lane 3). Thus, DNA was probably protected not due to its encapsulation within the polymer in the emulsion, but by the compaction of PLL, which accorded with our previous studies[13] that PLL can actually protect pDNA from being damaged in sonication, by its function to condense the DNA.[21]

Protection of the DNA structure during emulsification is important, as fragmentation of DNA could affect the transfection of NPs.[22] Although there was some open circular form of the DNA present in the stock (Fig. 2, lane 4), agarose gel electrophorsis results show that the encapsulation results in a partial transformation of DNA from the supercoiled to the open circular form (Fig. 2, lane 3). Similar transformation of the DNA following encapsulation has been reported by other investigators too.[23–25] However, this partial transformation of DNA is not expected to affect the transfectivity of NPs as it has demonstrated that the difference in the transfection levels of the supercoiled and relaxed forms of the DNA extracted from NPs is statistically insignificant.[24]

Stability in DNase I

Protection of pDNA from nucleases is one of the most crucial factors for efficient gene delivery *in vivo* as well as *in vitro*.[26] Complexation of DNA with cationic polymers is generally considered to prevent the condensed DNA from enzymatic breakdown. The extent of protection of pDNA from enzymatic degradation was investigated by incubating the naked DNA, PPD, NPs without PLL and PD in the presence of DNase I. Figure 3 (lanes 4 and 5) shows that when complexed with PLL, the condensed pDNA was efficiently protected in the NPs from the attack of DNase I. On the other hand, naked DNA (Fig. 3, lanes 2 and 3) and pDNA in NPs without PLL (Fig. 3, lanes 6 and 7) were rapidly degraded. It is all because of the condensation function of PLL, leading pDNA to construct a compact structure, which could give a full protection of DNA from the enzymatic attack. This result is consistent with the gel retardation result shown in Figure 3 (lanes 8 and 9).

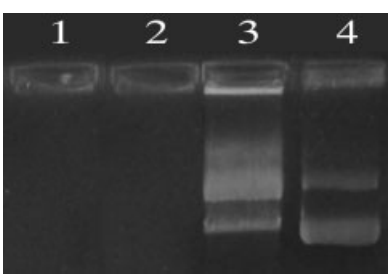

Figure 2 Agarose gel electrophorsis of DNA extracted from nanoparticles. Lane 1: naked DNA after sonification, Lane 2: extracted DNA from NPs without PLL, Lane 3: extracted DNA from PPD, and Lane 4: stock plasmid DNA as marker.

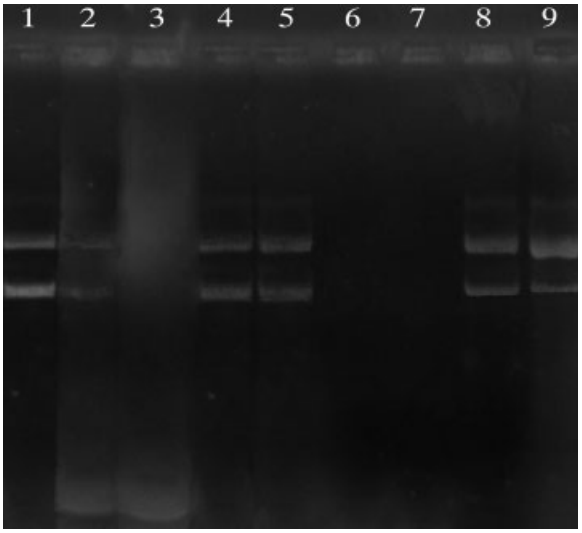

Figure 3 Agarose gel electrophoresis of naked DNA, PPD, NPs without PLL and PD subjected to DNase degradation. Lane 1: DNA marker; Lanes 2 and 3: Naked DNA incubated with DNase for 5 and 40 min, respectively; Lanes 4 and 5: PPD incubated with DNase for 5 and 40 min, respectively; Lanes 6 and 7: NPs without PLL incubated with DNase for 5 and 40 min, respectively; Lanes 8 and 9: PD incubated with DNase for 5 and 40 min, respectively.

X-gal staining and luciferase assay

Figure 4 demonstrates that PPD had rather high transfection efficiency both in HepG2 cells and Hela cells than NPs without PLL. The average transfection efficiencies were about 3% ± 0.9% and 1.2% ± 0.5% in HepG2 cells and Hela cells, respectively.

The transfection efficiency on HepG2 cells was determined by measuring the luciferase activity using luciferase assay system, as described earlier. In the case of the PELGE-NPs without PLL, very low luciferase activity was observed and the activity was similar to the control group, where naked pDNA was used. This was because the PELGE-NPs without PLL could not form a complex with the pDNA. PLL introduced in this system is essential, for its high affinity or condensation with DNA could protect DNA from ultrasonic radiation and DNase degradation, which increase the trasfection efficiency.

The transfection studies in HepG2 cells showed about three orders of magnitude higher luciferase protein levels for NPs with PLL when compared with the NPs without PLL ($P < 0.05$, $n = 6$) or the naked DNA (Fig. 5) for the same dose of NPs. The transfection studies in HepG2 cells could not be continued beyond 2 days because the cells reached confluence and began to detach.

Loaded in the NPs did not only be protected but also controlled released DNA into the medium. Because only 6–8 h were needed for commercialized lipofectamine™ contact with cells to be optimal transfection, while the transfection peak of NPs arrived at about 20-h contact. These results can be confirmed by similarly previous studies.[27]

This combination of taking both advantage of the neutral and cationic polymer is an attractive direct for the development of successful gene delivery. Other more ingenious systems were constructed recently,[21,28,29] which utilize the biocompatibility of PEG, buffering capacity of PMPA (or PAMAM or PEI) and high condensation function of PLL. When compared with them, the PPD delivery system does not have the segment with ''proton sponge effect,''

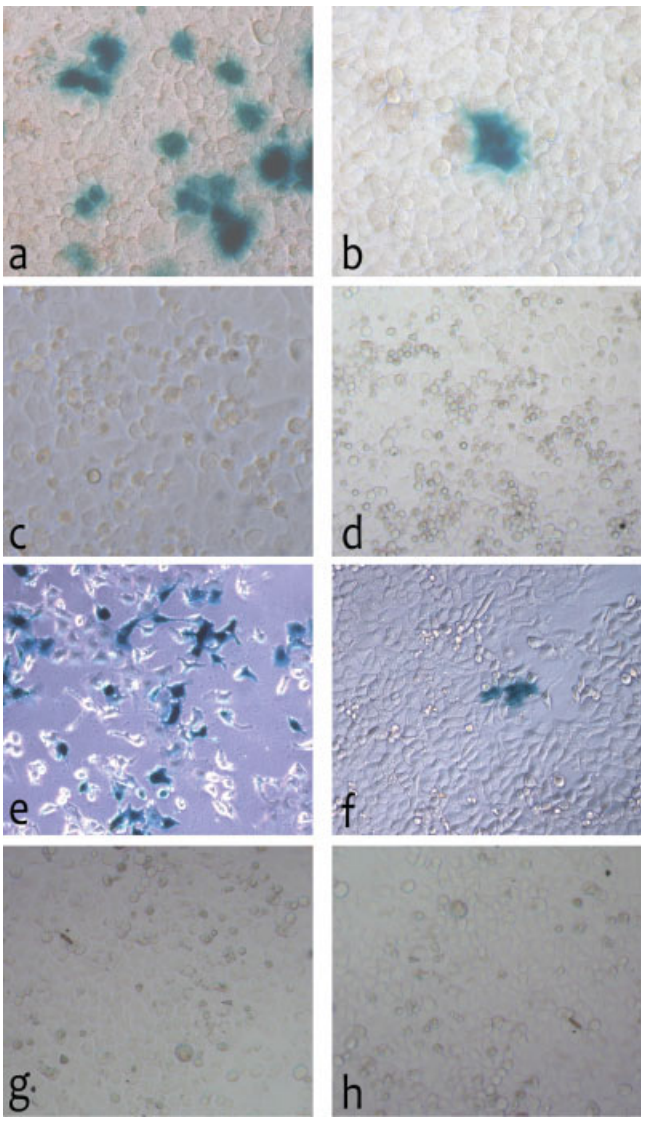

Figure 4 Transfection of pORF LacZ loaded PPDs on Hela (b) and hepatoma HepG2 (f) cells, compared with Lipofectamine (a and e), nanoparticles without PLL (c and g) and naked DNA (d and h). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

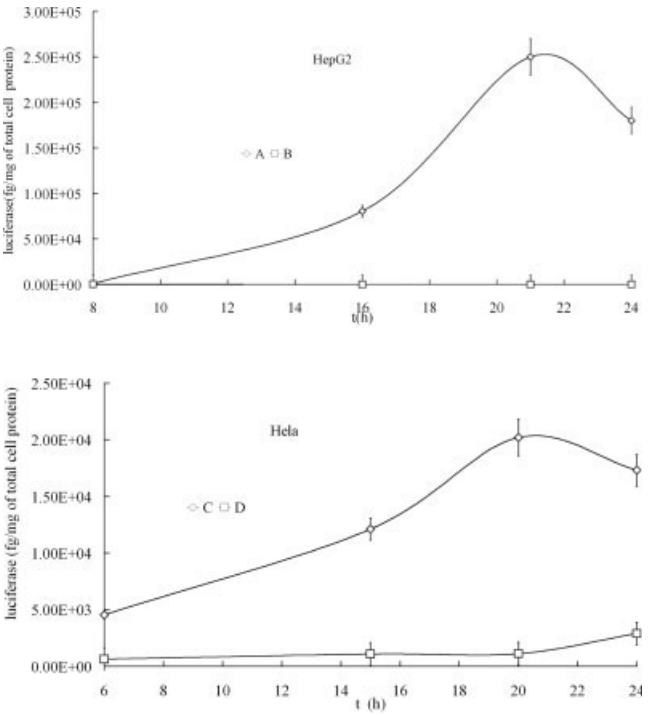

Figure 5 Transfection efficiency of nanoparticles in the HepG2 and Hela cell line ($n = 6$). The luciferase protein levels were measured 2 days after the transfection. The transfection time was designed at 8, 16, 21, and 24 h for HepG2, while 6, 15, 20, and 24 h for Hela. A and C represent the luciferase activity of PPD in HepG2 and Hela cells complexed with 2.5 μg of pDNA, while B and D represent the luciferase activity of NPs without PLL in HepG2 and Hela cells complexed with 2.5 μg of pDNA. The cells were seeded at 1×10^5 cells/well. Results are expressed as mean ± SD ($n = 6$).

but a more biocompatible part—PLGA. It was negatively charged under physiological condition, which may both less toxic *in vitro* and *in vivo*.[29]

CONCLUSION

In this research, preparation, characterization, and transfection efficiency of the novel gene delivery carrier composed of PELGE, PLL, and DNA (PPD) has been investigated. NPs were prepared by double emulsification-solvent evaporation technique with F68 as surfactant. Morphology and particle size of the new method prepared NPs were compared with the traditional ones. And then, the advantages of introduction of PLL in preparation were analyzed by antisonification and antinuclease properties of pDNA. Finally, cell transfection on Hela and HepG2 confirm the hypothesis that the introduction of polycationic peptide can better enhances the transfection. As the delivery system, PPD is a promising carrier, and so we will continue our research in this area by attaching cell targeting moieties to the PEG-modified carriers, followed by transfection studies using various cell lines *in vitro* and *in vivo* evaluation for the targeting of disease precaution and treatment in the future.

References

1. Hedley, M. L.; Curely, J.; Urban, T. Nat Med 1998, 4, 365.
2. Stuart, D. D.; Allen, T. M. Biochim Biophys Acta 2000, 1463, 219.
3. Prabha, S.; Zhou, W. Z.; Panyam, J.; Labhasetwar, V. Int J Pharm 2002, 244, 105.
4. Roy, K.; Mao, H. Q.; Huang, S. K.; Leong, K. W. Nat Med 1999, 5, 387.
5. Prabha, S.; Labhasetwar, V. Pharm Res 2004, 21, 354.
6. Labhasetwar, V.; Bonadio, J.; Goldstein, S.; Levy, R. J. Colloids Surf B 1999, 16, 28.
7. Duan, Y. R.; Nie, Y.; Gong, T.; Wang, Q.; Zhang, Z. R. J Appl Polym Sci 2006, 100, 1019.
8. Sun, X.; Duan, Y. R.; He, Q.; Lu, J.; Zhang, Z. R. Chem Pharm Bull 2005, 53, 599.
9. Sorgi, F. L.; Bhattacharya, S.; Huang, L. Gene Ther 1997, 4, 961.
10. Tsai, J. T.; Furstoss, K. J.; Michnick, T.; Sloane, D. L.; Paul, R. W. Biotechnol Appl Biochem 2002, 36, 13.
11. Capan, Y.; Woo, B. H.; Gebrekidan, S.; Ahmed, S.; DeLuca, P. P. J Control Release 1999, 60, 279.
12. Jeong, J. H.; Park, T. G. J Control Release 2002, 82, 159.
13. Nie, Y.; Duan, Y. R.; Zhang, Z. R. Drug Deliv 2006, 13, 441.
14. Wang, D. Q.; Robinson D. R.; Kwon G. S.; Samuel, J. J Control Release 1999, 57, 9.
15. Cui, Z.; Mumper, R. J. Bioconjugate Chem 2002, 13, 1319.
16. Swayam, P.; Labhasetwar, V. Pharm Res 2004, 21, 354.
17. Shakesheff, K. M.; Evora, C.; Soriano, I. I.; Langer, R. J Colloid Interf Sci 1997, 185, 538.
18. Sahoo, S. K.; Panyam, J.; Prabha, S.; Labhasetwar, V. J Control Release 2002, 82, 105.
19. Bathfield, M.; Graillat, C.; Hamaide, T. Macromol Chem Phys 2005, 206, 2284.
20. Kabanov, A. V.; Batrakova, E. V.; Alakhov, V. Y. J Control Release 2002, 82, 189.
21. Shigeto, F.; Kanjiro, M.; Nobuhiro, N.; Naoki, K.; Yuichi, Y.; Kazunori, K. J Am Chem Soc 2005, 127, 2810.
22. Walter, E.; Moelling, K.; Pavlovic, J.; Merkle, H. P. J Control Release 1999, 61, 361.
23. Ewert, K.; Ahmad, A.; Evans, H. M.; Schmidt, H. W.; Safinya, C. R. J Med Chem 2002, 45, 5023.
24. Cohen, H.; Levy, R. J.; Gao, J.; Fishbein, I.; Kousaev, V.; Sosnowski, S.; Slomkowski, S.; Golomb, G. Gene Ther 2000, 7, 1896.
25. Bergan, D.; Galbraith, T.; Sloane, D. L. Pharm Res 2000, 17, 967.
26. Jeong, J. H.; Park, T. G. J Control Release 2002, 82, 159.
27. Kim, I. S.; Lee, S. K.; Park, Y. M.; Lee, Y. B.; Shin, S. C.; Lee, K. C. Int J Pharm 2005, 298, 255.
28. Atsushi, H.; Masanori, K.; Takashi, M.; Toshinari, T.; Kenji, K. Bioconjugate Chem 2006, 17, 3.
29. Thomas, M.; Klaus, K.; Holger, P.; Udo, B.; Karl, H. V.; Jindrich, K.; Thomas, K. Bioconjugate Chem 2005, 16, 785.