International Journal of Pharmaceutics 392 (2010) 224–231

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Ternary nanoparticles of anionic lipid nanoparticles/protamine/DNA for gene delivery

Hong Yuan*, Wei Zhang, Yong-Zhong Du, Fu-Qiang Hu

College of Pharmaceutical Science, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, PR China

ARTICLE INFO

Article history:
Received 1 November 2009
Received in revised form 8 February 2010
Accepted 9 March 2010
Available online 15 March 2010

Keywords:
Lipid nanoparticles
Protamine
Plasmid DNA
Ternary nanoparticles
Gene transfection

ABSTRACT

In this study, the lipid nanoparticles with excellent cellular uptake capacity were utilized to prepare lipid nanoparticles/protamine/DNA ternary nanoparticles for gene delivery. Anionic lipid nanoparticles consisting of monostearin (MS) and different content of oleic acid (OA) were prepared. The lipid nanoparticles had an average size ranging from 23.6 to 71.3 nm and a zeta potential about −30 mV. The protamine/DNA complex was prepared by mixing the DNA and protamine solution with 1:2 mass ratio. The average size of protamine/DNA complex was 128.5 nm and with 19.4 mV zeta potential. Lipid nanoparticles/protamine/DNA ternary nanoparticles were then prepared by combining the protamine/DNA binary complex with lipid nanoparticles. The ternary nanoparticles had an average size ranging from 192.7 to 260.6 nm and were negatively charged. Gene transfection efficiency was improved by increasing OA content in lipid nanoparticles. Results of cellular uptake showed that the uptake ability of lipid nanoparticles was enhanced by increasing the OA content. Lipid nanoparticles with 20 wt% OA/protamine/DNA ternary nanoparticles (w/w/w, 65:6:3) showed the best and the most durable gene transfection even in the presence of serum.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Gene therapy is a promising treatment for some genetic-based and infectious diseases (Anas El-Aneed, 2004), and lots of efforts have been made for developing an efficient gene transfection vector system.

Historically, there are mainly two categories of vector systems: viral and non-viral. The use of viruses is a powerful technique, but accompanies a series of side effects, oncogenicity and safety issues. Non-viral vectors have shown low immunogenicity, and the capacity to carry large inserts, which may be more suitable for clinical use. However, the transfection efficiency remained very low in comparison to conventional viral agents (Lonez et al., 2008; Morille et al., 2008).

Non-viral vectors are mainly composed of cationic polymers and cationic lipids such as polyethyleneimine (PEI) (Breunig et al., 2005), poly (L-lysine) (PLL) (Choi et al., 2007), chitosan (Romoren et al., 2002), 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) (Choi et al., 2008), 1,2-dioleyl-3-trimethylammonium-propane (DOTAP) (Vaysse et al., 2006) and so on. Some cationic lipid-based gene delivery systems are also explored later, including liposomes, emulsions and lipid nanoparticles. Lipid nanoparticles include solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). SLN have been introduced into the applications in the cosmetic industry and pharmaceutical researches in the last decades (Müller et al., 1995; Trotta et al., 2003; Shidhaye et al., 2008). NLC were then developed based on the SLN by incorporating liquid lipids or spatially different lipids in the solid core of the particles. Gene transfections using cationic liposomes and cationic lipid emulsions have been widely reported (Liu et al., 1996; Kim et al., 2002). However, there are only a few reports on lipid nanoparticles for gene delivery (Olbrich et al., 2001; Tabatt et al., 2004; Rudolph et al., 2004).

The disadvantages of non-viral gene carrier systems are their low transfection efficiency, toxicity (linked to their positive charge) and lack of sustainable gene expression. These disadvantages make non-viral gene carrier systems difficult to be used in clinical settings. Some improvements have been made by condensing DNA and then mixing them with liposomes (Junghans et al., 2005b; Sun et al., 2005). DNA condensation was critical for efficient gene transfections because it protected DNA from enzyme degradation and subsequently made the vector system more stable in the presence of serum (Lee and Huang, 1996; Junghans et al., 2005a).

Although cationic lipids have several advantages, cytotoxicity remains a problem especially *in vivo*. This may result from the positive charge of the vector. In this study, anionic lipid nanoparticles with different content of oleic acid (0–20 wt%) were prepared by the aqueous solvent diffusion method. Monostearin (MS) was chosen as solid lipids for its relative higher cellular uptake than some other lipid components (Yuan et al., 2008). Poloxamer188 (0.1%,

* Corresponding author. Tel.: +86 571 88208439; fax: +86 571 88208439.
E-mail address: yuanhong70@zju.edu.cn (H. Yuan).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.03.025

Table 1
The size and zeta potential of different lipid nanoparticles and their respective ternary nanoparticles.

Samples	Number average size (nm)	Zeta potential (mV)
Protamine/DNA binary complex	128.5 ± 18.6	19.4 ± 0.6
LN with 0 wt% OA	54.2 ± 13.8	−23.9 ± 1.2
LN with 5 wt% OA	72.3 ± 5.7	−31.1 ± 1.6
LN with 10 wt% OA	23.6 ± 2.6	−30.1 ± 0.5
LN with 20 wt% OA	26.7 ± 5.1	−31.3 ± 1.0
LN with 0 wt% OA/protamine/DNA (65:6:3, w/w/w)	260.6 ± 21.2	−16.2 ± 0.1
LN with 5 wt% OA/protamine/DNA (65:6:3, w/w/w)	196.5 ± 32.5	−18.4 ± 0.5
LN with 10 wt% OA/protamine/DNA (65:6:3, w/w/w)	192.7 ± 24.2	−19.6 ± 4.5
LN with 20 wt% OA/protamine/DNA (65:6:3, w/w/w)	197.6 ± 14.4	−17.1 ± 4.8

w/v) solution was selected as an aqueous phase. It was shown that poloxamer 188 could stabilize some liposome systems and increase gene delivery capacity (Law et al., 2006). In our study, plasmid DNA was mixed with protamine for condensation, and then incubated with lipid nanoparticles to prepare ternary nanoparticles for gene delivery. It has been reported that the addition of protamine could enhance the efficiency of gene transfections (Balhorn et al., 2000; Fujita et al., 2008), and the nuclear localization signals (NLS) in protamine molecule could orientate DNA to the nucleus of cells (Gao and Huang, 1996; Ziemienowicz et al., 1999). The gel retardation and Deoxyribonuclease I (DNase I) protection were carried out to evaluate the condensing ability of protamine. The cytotoxicity was assessed by MTT (5-diphenyl-tetrazolium bromide) assay in human embryonic kidney (HEK293) cells. The gene transfection was conducted using pEGFP and luciferase expression gene as reporter genes. The cellular uptake was performed to evaluate its effect on gene transfection.

2. Materials and methods

2.1. Materials

Monostearin (MS) was provided by Shanghai Chemical Reagent Co. Ltd., China. Poloxamer 188 was purchased from Shenyang Jiqi Pharmaceutical Co. Ltd., China. Lipofectamine^{TM} 2000 was from Invitrogen Corporation, USA. Protamine sulfate was purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Hoechst33342 used as staining reagents for nucleuses was from Sigma Chem. Co., St. Louis, USA. Octadecylamine (ODA) was purchased from Fluka, USA and fluorescein isothiocyanate (FITC) was purchased from Acros Organic, USA for the preparation of ODA-FITC. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA) for cytotoxicity assay. Deoxyribonuclease I (DNase I) was from Takara Biotechnology (Dalian) Co., Ltd. BCA Protein Assay Kit was purchased from Beyotime Institute of Biotechnology, Jiangsu, China. The luciferase assay agent was purchased from Chemi&BioLuminescence, Shanghai, China. PEGFP-N1 (4.7 kb), luciferase gene and human embryonic kidney (HEK293) cells were gifted from National Institute of Health, USA. Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Co., Ltd. (Zhejiang, China). Dulbecco's modified eagle's medium (DMEM) was purchased from GibcoBRL (Gaithersberg, MD, USA). Ethanol and other chemicals were analytical reagent grade.

2.2. Preparation of anionic lipid nanoparticles

Lipid nanoparticles (LN) with 0 wt%, 5 wt%, 10 wt%, and 20 wt% content of oleic acid were prepared by the aqueous solvent diffusion method reported in our previous study (Hu et al., 2005). Thirty mg monostearin and 0 mg, 1.5 mg, 3 mg or 6 mg oleic acid were mixed in 3 mL ethanol for the preparation of 0 wt%, 5 wt%, 10 wt%, 20 wt% LN, respectively. The organic solution was then dispersed into 30 mL poloxamer 188 solution (0.1%, w/v) agitating at 400 rpm in the 60 °C water bath for approximately 5 min. It was then cooled at room temperature. The dispersion was frozen at −70 °C to precipitate LN. After collection by centrifugation (20,000 rpm for 15 min), lipid nanoparticles were re-dispersed in 30 mL poloxamer 188 solution (0.1%, w/v).

2.3. Preparation and characterization of ternary nanoparticles

The ternary system was prepared using methods reported before (Birchall et al., 2000). Protamine/DNA binary complex was prepared by mixing 0.5 mg/mL protamine with 0.5 mg/mL DNA solution at room temperature for 15 min. The ternary nanoparticles consisting of LN/protamine/DNA were prepared by sequential addition of lipid nanoparticles to protamine/DNA binary complex at proper ratios. The average diameter and zeta potential of ternary nanoparticles were measured by dynamic light scattering using a zetasizer (3000HS, Malvern Instruments Ltd., UK).

The surface morphology of nanoparticles was further examined by transmission electronic microscopy (JEM-1230EX, Japan). Binary complex and ternary nanoparticles suspensions were dropped onto

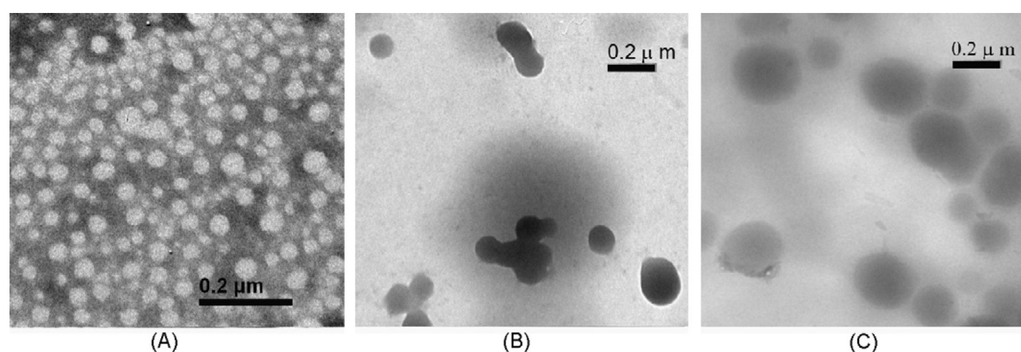


Fig. 1. TEM images of lipid nanoparticles with 20wt% OA, the protamine/DNA binary complex (weight ratio of protamine to DNA was 2:1) and lipid nanoparticles/protamine/DNA ternary nanoparticles (65:6:3, w/w/w).

Table 2
IC_{50} of lipid nanoparticles on HEK293 cell lines and MCF-7 cell lines by the MTT assay.

Samples	IC_{50} (μg/mL) on HEK293	IC_{50} (μg/mL) on MCF-7
LN with 0 wt% OA	320.8 ± 41.5	310.5 ± 1.8
LN with 5 wt% OA	329.4 ± 12.1	390.0 ± 9.1
LN with 10 wt% OA	323.6 ± 13.4	370.4 ± 2.5
LN with 20 wt% OA	322.1 ± 6.01	334.3 ± 20.9

the copper grids and lipid nanoparticles suspensions were further stained with 2% aqueous solution of sodium phosphotungstate for negative stain. The air-dried samples were then directly observed under the transmission electronic microscopy.

2.4. Cytotoxicity of lipid nanoparticles

The cytotoxicity of lipid nanoparticles was evaluated using HEK293 cells and MCF-7 cells (Human breast adenocarcinoma cell line) by the MTT assay. Cells were cultured in a 24-well plate at the density of 1×10^5 cells/mL. After incubation for 24 h, the media were exchanged with 1 mL culture medium containing different concentrations of lipid nanoparticles with 0 wt%, 5 wt%, 10 wt% and 20 wt% content of OA. Controlled experiments were carried out using the complete growth culture medium only. After incubation for 72 h, 50 μL MTT solution (5 mg/mL in PBS) was then added to each well. The medium was removed after 4 h. Then, the crystals were solubilized with 1 mL DMSO and then detected by a microplate reader (BioRad, Model 680, USA) at 570 nm. Survival percentage was calculated as compared to controlled cells (100% survival). We then calculated the inhibition concentration of 50% cells (IC_{50}) using the survival percentages at different concentrations of LN according to the report by Mosmann (1983).

HEK293 cells and MCF-7 cells were also treated with protamine/DNA binary complex, ternary nanoparticles and lipofectamine™ 2000/DNA complex for 72 h, and the cell viability was measured by MTT assay. All the experiments were performed in triplicate.

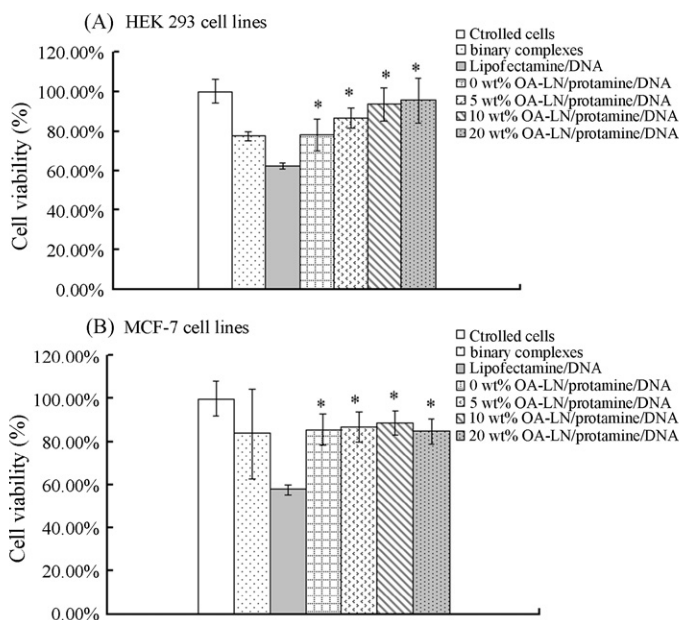


Fig. 2. Cell viabilities of binary complex, lipofectamine/DNA complex, LN/protamine/DNA nanoparticles in HEK293 cell lines and MCF-7 cell lines ($n = 3$) (*$p < 0.05$ compared with the cell viability of lipofectamine/DNA complex, *t*-test).

2.5. Gel retardation assay

To evaluate the condensation capability of binary complex (different weight ratios of protamine to DNA) and ternary nanoparticles (different amounts of lipid nanoparticles), agarose gel electrophoresis (0.8%, w/v) were carried out under a voltage of 120 V for 30 min. Images were obtained using a UV transilluminator and a digital imaging system (GL 200, Kodak, USA).

2.6. DNase I protection assay

DNase I was added to binary complex and ternary nanoparticles to obtain a final concentration of 1 U DNase I/2.5 μg DNA, and the mixtures were incubated at 37 °C for 30 min followed by adding 0.5 M EDTA to terminate the reaction. Afterwards, 10% SDS solution was added to the samples to obtain a final concentration of 1% to release DNA from protamine or lipid nanoparticles. Samples were then analyzed by electrophoresis on the agarose gel and the integrity of the DNA in each sample was compared to naked DNA treated with DNase I.

2.7. In vitro gene transfection

Luciferase gene and pEGFP-N1 were used to evaluate the efficiency of gene transfections. HEK293 cells and MCF-7 cells were seeded in 24-well plates at a density of 1×10^5 cells/well in 1 mL of complete medium. After incubation for 24 h, the medium was replaced with 0.5 mL 10%-FBS medium or serum-free medium containing various concentrations of ternary nanoparticles. The medium was then replaced with 1 mL of fresh complete medium after six hours' incubation. Luciferase gene expression was assessed by measuring luciferase activity in cell extracts using chemiluminescence detector (FB12, Sirius, Berthold Dection System, Germany). The relative light units (RLU) were normalized to protein concentrations in the cell extracts measured by the BCA method using an ELISA plate reader (BioRad, Model 680, USA). Gene transfection tests with lipofectamine™ 2000/DNA complex were performed as positive controls according to the manufacture's protocol. Cells transfected with pEGFP-N1 were also directly examined under a fluorescence microscope (Olympus America, Melville, NY).

2.8. Cellular uptake of lipid nanoparticles

HEK293 cells were seeded at a density of 1×10^5 cells/well in 1 mL of growth medium. Cells were then incubated with ODA–FITC labeled lipid nanoparticles with 5 wt%, 10 wt% and 20 wt% content of OA suspension (prepared as reported before (Yuan et al., 2007)) at the concentration of 100 μg/mL for different incubation duration of 1 h, 3 h, 6 h, 12 h, 24 h. After washing the cells with PBS three times, 100 μL cell lysate (1 mg/mL) was added to each well and incubated for 5 min, and then the cells were harvested by adding 1 mL PBS. Finally, the cell lysate was centrifuged at 10,000 rpm for 10 min, and the supernatant was supplied to fluorescence assay by using fluorometer (F-4000, HITACHI Co., Japan) (excitation: 495 nm; emission: 517 nm). The cellular uptake percentages of fluorescent lipid nanoparticles were normalized by protein amount, which was determined by BCA protein Assay Kit (Beyotime biotechnology, China). The cellular uptake percentages of fluorescent lipid nanoparticles were calculated from the following equation:

$$\text{Cellular uptake percentage (\%)} = \frac{\text{Fluorescent value of the sample/protein amount of the sample}}{\text{Fluorescent value of total LN/protein amount of controlled group}} \times 100$$

Fluorescent images of cells incubated with ODA-FITC labeled lipid nanoparticles were examined under a fluorescence microscope (OLYMPUS America, Melville, NY) at 1 h, 3 h, 6 h, 12 h, 24 h.

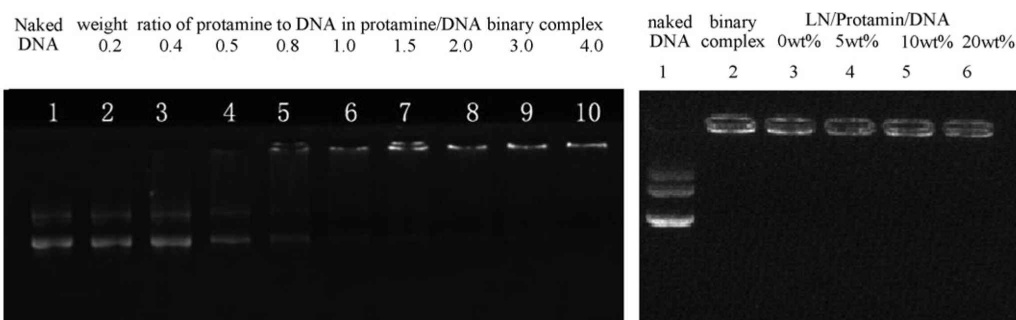


Fig. 3. Gel retardation assay of protamine/DNA complex (A) and LN/protamine/DNA (B). For B, lane 1 is naked DNA; lane 2 is protamine/DNA complex (w/w, 2:1); lanes 3–6 are lipid nanoparticles with 0 wt%, 5 wt%, 10 wt% and 20 wt% content OA/protamine/DNA nanoparticles (w/w/w, 3:2:1), respectively.

2.9. Statistical analysis

Results are reported as means ± standard deviation (S.D.). Paired t-test was applied to assess the statistical difference among different groups. Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Preparation and characterization of ternary nanoparticles

In this study, lipid nanoparticles were prepared using MS and different content of OA in poloxamer 188 solution. The size and zeta potential were measured using a zetasizer. As shown in Table 1, the binary complex of protamine/DNA had a size of 128.5 ± 18.6 nm. LN with 0 wt% content of OA had an average size of 54.2 ± 13.8 nm. The sizes of LN with 10 wt% and 20 wt% content of OA were smaller than that of LN with 5 wt% content of OA. The zeta potential of protamine/DNA binary complex (w/w, 2:1) was 19.4 ± 0.6 mV, while lipid nanoparticles and their corresponding ternary nanoparticles were negatively charged (Table 1). The protamine/DNA binary complex formed by electrostatic interactions between the positive arginine-rich domain of protamine and the negative major groove of DNA. Anionic lipid nanoparticles may further interacted with positively charged protamine/DNA binary complex to form the ternary nanoparticles for gene transfections.

TEM images of lipid nanoparticles with 20 wt% OA, the protamine/DNA binary complex, lipid nanoparticles/protamine/DNA ternary nanoparticles were observed under a transmission electronic microscopy (JEM-1230EX, Japan). The lipid nanoparticles with 20 wt% OA formed regularly spherical particles (Fig. 1A). Adding lipid nanoparticles to the protamine/DNA binary complex (Fig. 1B) caused the formation of ternary nanoparticles (Fig. 1C) which were about 200 nm in diameters.

3.2. Cellular toxicity

The IC_{50} of lipid nanoparticles was all above 300 μg/mL at incubation time of 72 h (Table 2) on both HEK293 cells and MCF-7 cells, which suggested that lipid nanoparticles were safe gene carriers.

HEK293 cells and MCF-7 cells were treated with protamine/DNA binary complex, ternary nanoparticles and lipofectamine^{TM} 2000/DNA complex for 72 h, and the cell viability was evaluated by MTT assay. Compared to lipofectamine^{TM} 2000/DNA complex, ternary nanoparticles exhibited significantly higher cell viability ($p < 0.05$, t-test) as shown in both HEK293 cells and MCF-7 cells (Fig. 2).

Cationic lipid can interact with enzymes such as protein kinase C (PKC) and inhibit its activity, thus leads to cytotoxicity (Fischer et al., 1999). Cationic polymer such as PEI and PLL may interact with negatively charged serum protein, which precipitated in large

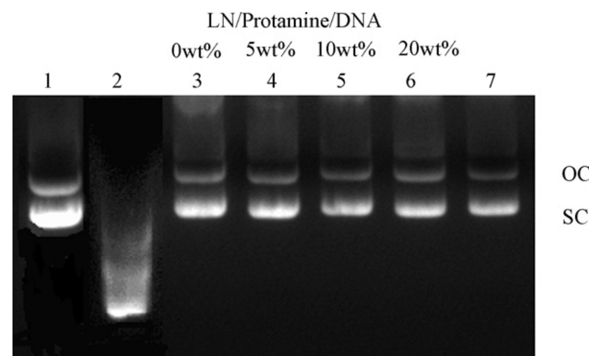


Fig. 4. Agarose gel electrophoresis of DNA extracted from different vectors after being incubated with DNase I. Lane 1 is naked DNA without any treatment; lane 2 is naked DNA treated with DNase I for 30 min; lanes 3–6 are DNA extracted from 0 wt%, 5 wt%, 10 wt% and 20 wt% OA-LN/protamine/DNA nanoparticles (w/w/w, 3:2:1) treated with DNase I for 30 min, respectively; lane 7 is for DNA extracted from protamine/DNA complex (w/w, 2:1) (OC stands for open circular, SC stands for super coil).

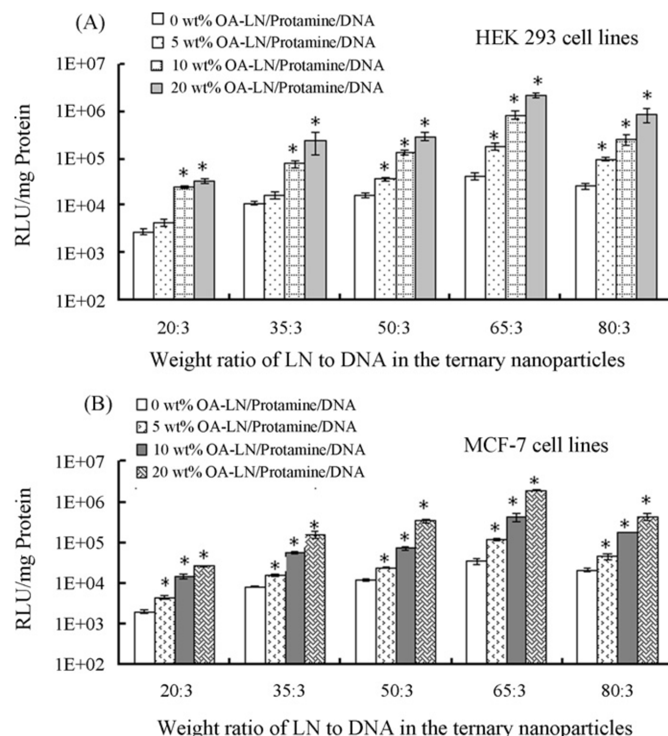


Fig. 5. Gene transfection of ternary nanoparticles on both HEK293 and MCF-7 cells at the transfection time of 72 h (*$p < 0.05$ compared with that of 0 wt% OA-LN/protamine/DNA at the same weight ratio).

clusters and adhered to cell surfaces. When administered into circulatory system, they are rapidly bound to plasma proteins and cleared from the circulation (Fischer et al., 1999; Ward et al., 2001). However, the overall zeta potentials of the ternary nanoparticles prepared in this study were all negatively charged, which may avoid the toxicity resulted from cationic vectors.

3.3. Gel retardation assay

Gel retardation assay was carried out to evaluate the condensing ability of protamine/DNA binary complex and lipid nanoparticles/protamine/DNA. The complete retardation of the binary complex can be observed when the weight ratio of protamine to DNA was above 1.0 (Fig. 3A).

In preliminary trials, we found that anionic lipid nanoparticles could condense protamine/DNA binary complex (w/w, 2:1) while the weight ratio of lipid nanoparticles to DNA exceeded 3 (Fig. 3B).

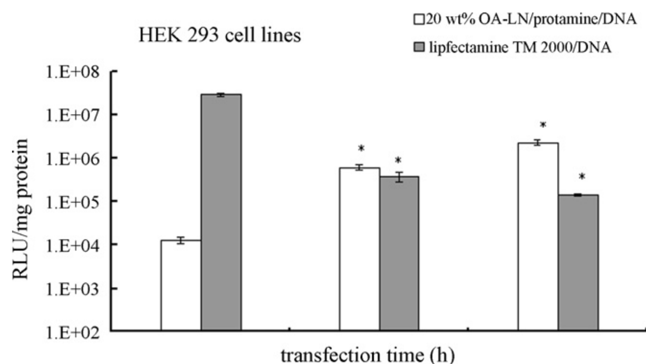
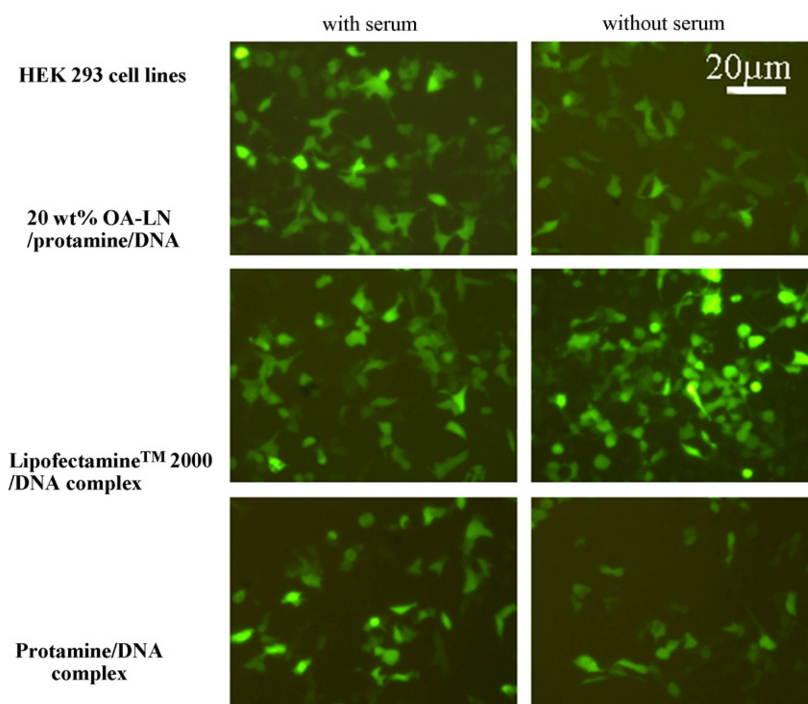


Fig. 6. Effect of post-transfection time on transfection of lipofectamine™ 2000/DNA complex and 20 wt% OA-LN/protamine/DNA nanoparticles on HEK293 cell lines (*$p < 0.05$ was against to transfection efficiencies of 20 wt% OA-LN/protamine/DNA or lipofectamine™ 2000/DNA complex at the post-transfection time of 24 h, respectively).



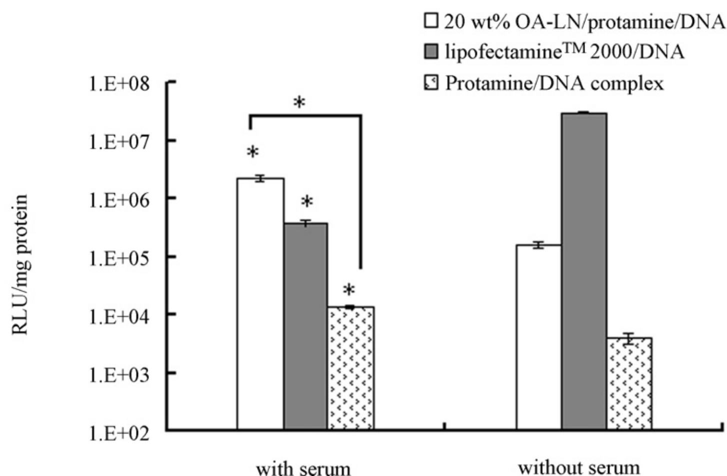


Fig. 7. Effect of serum on transfection of 20 wt% OA-LN/protamine/DNA nanoparticles, lipofectamine™ 2000/DNA complex and protamine/DNA complex on HEK293 cell lines (*$p < 0.05$ compared with transfection efficiencies of 20 wt% OA-LN/protamine/DNA, lipofectamine™ 2000/DNA complex, binary complex without serum, respectively, and the efficiency of LN/protamine/DNA was also against to that of binary complex when with serum).

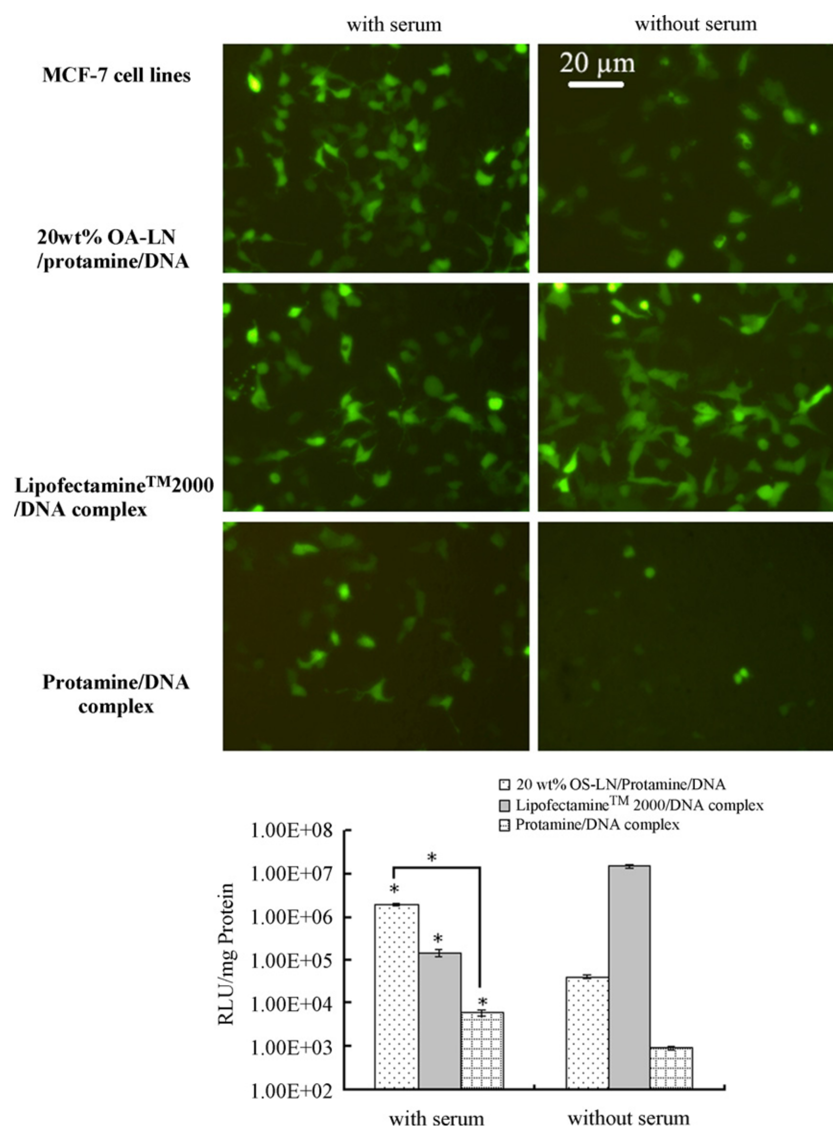


Fig. 8. Effect of serum on transfection of 20 wt% OA-LN/protamine/DNA nanoparticles, lipofectamine^{TM} 2000/DNA complex and protamine/DNA complex on MCF-7 cell lines (*$p < 0.05$ compared with transfection efficiencies of 20 wt% OA-LN/protamine/DNA, lipofectamine^{TM} 2000/DNA complex, binary complex without serum, respectively, and the efficiency of LN/protamine/DNA was also against to that of binary complex when with serum).

3.4. DNase I protection assay

One of the major barriers for gene efficiency *in vitro* and *in vivo* is the degradation of plasmid DNA by nucleases, e.g., DNase I, DNase II, etc.

DNase I was used as a model enzyme to detect whether or not the ternary nanoparticles could protect plasmid DNA from nuclease digestion. Protamine/DNA binary complex, LN/protamine/DNA nanoparticles were exposed to DNase I for 30 min. Naked DNA treated with DNase I was completely fragmented (as shown in Fig. 4) after 30-min incubation, while binary complex and ternary nanoparticles efficiently protected DNA form enzyme degradation (Fig. 4).

The presence of DNA topology was also investigated using agarose gel electrophoresis. Naked DNA not treated with DNase I (Lane 1 in Fig. 4) presented two bands, the upper circular form (OC) and the lower supercoiled from (SC). When naked DNA was incubated with DNase I, just fragments with different lengths of base pair were seen. However, binary complex and ternary nanoparticles could protect the plasmid DNA from degradation and both the OC and SC patterns of DNA could be seen by agarose gel electrophoresis.

3.5. In vitro gene transfection test

The intracellular expression of DNA (luciferase genes) was measured as relative light unit (RLU) and the results were normalized by protein content. The effects of the concentration of lipid nanoparticles, content of OA in lipid nanoparticles, post-tansfection time and serum on the gene transfection of HEK 293 cells were investigated. PEGFP-N1 was used as the other model plasmid to evaluate the efficiency of gene transfection and cells transfected with pEGFP-N1 loading vectors were directly examined under a fluorescence microscope (Olympus America, Melville, NY).

To determine the optimal ratio of lipid nanoparticles to DNA for high transfection efficiency, various concentrations of lipid nanoparticles and a constant concentration (3 μg/mL) of plas-

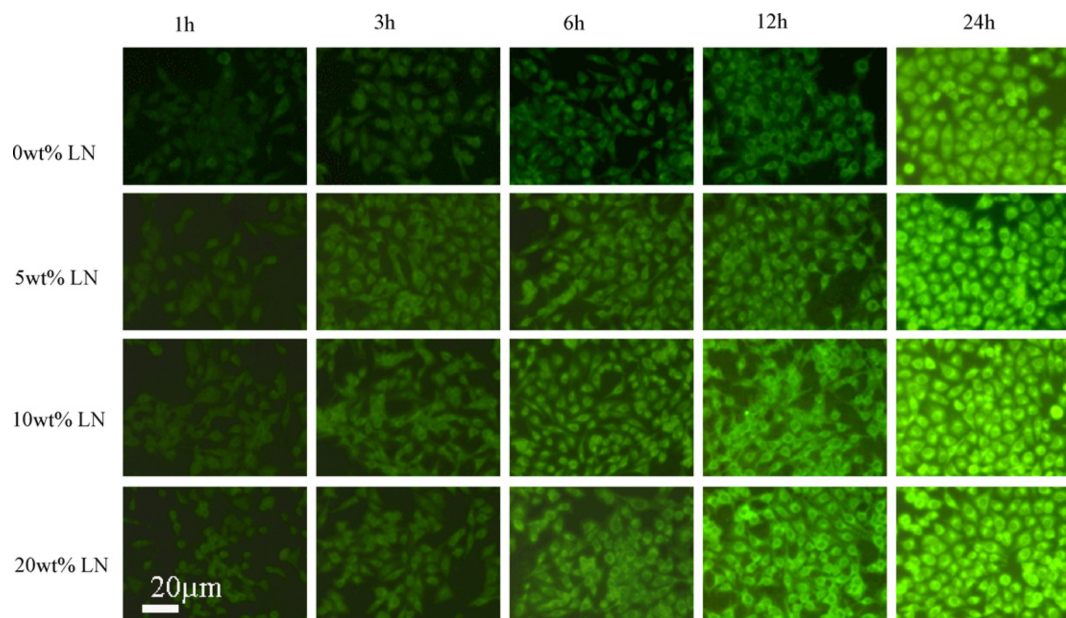


Fig. 9. Fluorescent images of HEK293 cell incubated with ODA-FITC labeled lipid nanoparticles at different time intervals (the concentration of lipid nanoparticles are 100 μg/mL).

mid DNA and protamine (6 μg/mL) were used for luciferase gene expression. While the weight ratio of lipid nanoparticles to DNA reached 65:3 (protamine/DNA = 6:3, w/w), the ternary nanoparticles demonstrated the best gene transfection on both HEK293 and MCF-7 cells (Fig. 5). Data also showed that lipid nanoparticles with OA achieved higher efficiency in gene transfection than that of lipid nanoparticles without OA. The gene transfection efficiency was the highest as the OA content reached 20 wt% compared to other lipid nanoparticles with lower OA content ($p < 0.05$, t-test). Lipid nanoparticles with 20 wt% content of OA were therefore selected as the optimal formulation.

Using selected lipid nanoparticles with 20 wt% content of OA, we compared the effect of post-transfection time on transfections of LN/protamine/DNA nanoparticles. Lipofectamine™ 2000/DNA complex was used as a positive control. Fig. 6 shows that the transfection efficiency of lipofectamine™ 2000/DNA complex was highest at 24 h post-tansfection, and decreased rapidly at 48 h and 72 h post-transfection. On the other hand, the transfection efficiency of 20 wt% OA-LN/protamine/DNA nanoparticles was highest at 72 h post-transfection time. It appeared that the gene expression of LN/protamine/DNA nanoparticles was more durable than that of lipofectamine™ 2000/DNA complex.

The effect of serum on gene transfections of LN/protamine/DNA nanoparticles and lipofectamine™ 2000/DNA complex was evaluated. As shown in Fig. 7 (HEK293 cell lines) and Fig. 8 (MCF-7 cell lines), the gene transfection efficiency of lipofectamine™ was significantly decreased ($p < 0.05$, t-test) in the presence of the medium containing 10% serum, while the level of transfections for binary complex and ternary nanoparticles was increased ($p < 0.05$, t-test). The gene transfection efficiency of ternary nanoparticles was greatly enhanced by incorporated lipid nanoparticles to the binary complex ($p < 0.05$, t-test). The possible explanation is that cells may be more viable under the circumstance with 10% serum medium. Furthermore, the pre-condensation of DNA by protamine (Junghans et al., 2005a, 2005b) and the use of poloxamer 188 (Law et al., 2006) as the re-dispersed solution for LN preparation may contribute to the relative more stable status of LN/protamine/DNA nanoparticles than that of lipofectamine™ 2000/DNA complex in the serum-containing medium.

3.6. Cellular uptake of lipid nanoparticles

HEK 293 cells were used for the cellular uptake. Lipid nanoparticles with various content of OA were labeled with ODA-FITC. After incubating with ODA-FITC-labeled lipid nanoparticles for 1 h, 3 h, 6 h, 12 h and 24 h, cells were examined under a fluorescent microscopy. Cell lysates were then collected for fluorescence detection.

As shown in Fig. 9, lipid nanoparticles with OA were incorporated into HEK293 cells more quickly than those without OA, and the cellular uptake capacity was greater when the content of OA increased. Quantitative cellular uptake further confirmed what was observed from fluorescent images (Fig. 10). This result was in agreement with that from gene transfection assay. The lipid nanoparticles with 20 wt% OA can be effectively incorporated into cell membranes and facilitate the delivery of genetic materials into cells.

The addition of liquid oleic acid to the lipid matrix of lipid nanoparticles enhanced cellular uptake, and the cell uptake capacity increased as the content of OA increased (20 wt%-OA LN > 10 wt%-OA LN > 5 wt%-OA LN). It has been reported that the affinity between fatty acid and cell membrane was related to the

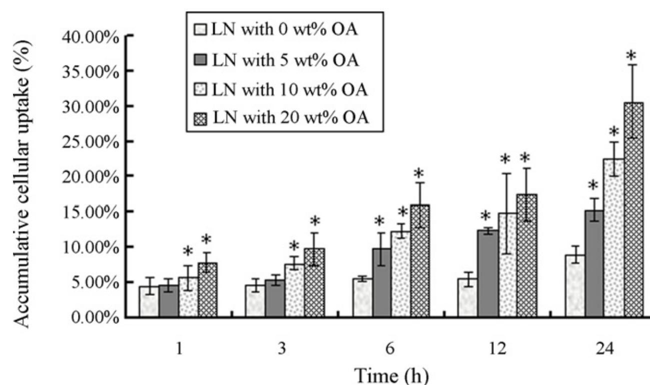


Fig. 10. Cellular uptake percentages of lipid nanoparticles on HEK293 cell at different incubation times (*$p < 0.05$ was against to cellular uptake of lipid nanoparticles with 0 wt% content of OA at the same time interval, respectively).

length of the carbon chain and the degree of saturation of the fatty acid. Oleic acid, an unsaturated fatty acid, may show higher affinity to cell membrane than that of saturated fatty acid (Tranchant et al., 1997). Furthermore, the fusing point of mixed lipid was lower than that of singular lipid, and cells were more likely to uptake materials with lower fusing point (Gualbert et al., 2003).

4. Conclusion

Lipid nanoparticles using MS and different amounts of OA in the organic phase were prepared by solvent diffusion method. In this research, LN/protamine/DNA ternary gene delivery systems were applied. The cellular toxicity of lipid nanoparticles was very low and IC_{50} were all about 300 μg/mL. It showed that addition of OA to the lipid matrix enhanced cellular uptake and increased gene transfection efficiency. Our results indicated that lipid nanoparticles with 20 wt%OA/protamine/DNA was an effective non-viral gene transfer vector even in the presence of serum, and its gene expression was durable as long as 72 h, which may be optimal for future *in vivo* application of this gene delivery system.

Acknowledgment

This work was sponsored by Zhejiang Provincial Program for the Cultivation of High-level Innovative Health talents.

References

Anas El-Aneed, 2004. Current strategies in cancer gene therapy. Eur. J. Pharmacol. 498, 1–8.

Balhorn, R., Brewer, L., Corzett, M., 2000. DNA condensation by protamine and arginine-rich peptides: analysis of toroid stability using single DNA molecules. Mol. Reprod. Dev. 56, 230–234.

Birchall, J.C., Kellaway, I.W., Cumbleton, M., 2000. Physical stability and in-vitro gene expression efficiency of nebulised lipid–peptide–DNA complexes. Int. J. Pharmaceut. 197, 221–231.

Breunig, M., Lungwitx, U., Liebl, R., Fontanari, C., Klar, J., Kurtz, A., et al., 2005. Gene delivery with low molecular weight linear polyethylenimines. J. Gene Med. 7, 1287–1298.

Choi, Y.R., Chae, S.Y., Ahn, C.H., Lee, M., Oh, S., Byun, Y., Rhee, B.D., Ko, K.S., 2007. Development of polymeric gene delivery carriers: PEGylated copolymers of L-lysine and L-phenylalanine. J. Drug Target. 15, 391–398.

Choi, S.H., Jin, S.E., Lee, M.K., Lim, S.J., Park, J.S., Kim, B.G., Ahn, W.S., Kim, C.K., 2008. Novel cationic solid lipid nanoparticles enhanced p53 gene transfer to lung cancer cells. Eur. J. Pharm. Biopharm. 68, 545–554.

Fischer, D., Bieber, T., Li, Y., Elsasser, H.P., Kissel, T., 1999. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm. Res. 16, 1273–1279.

Fujita, T., Furuhata, M., Hattori, Y., Kawakami, H., Toma, K., Maitani, Y., 2008. High gene delivery in tumor by intratumoral injection of tetraarginine-PEG lipid-coated protamine/DNA. J. Control. Rel. 129, 124–127.

Gao, X., Huang, L., 1996. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry 35, 1027–1036.

Gualbert, J., Shahgaldian, P., Cloleman, A.W., 2003. Interactions of amphiphilic calix(4)arene-based solid lipid nanoparticles with bovine serum albumin. Adv. Drug Deliv. Tev. 253, 23–38.

Hu, F.-Q., Jiang, S.-P., Du, Y.-Z., Yuan, H., Ye, Y.-Q., Zeng, S., 2005. Preparation and characterization of stearic acid nanostructured lipid carriers by solvent diffusion method in an aqueous system. Colloids Surf. B: Biointerf. 45, 167–173.

Junghans, M., Loitsch, S.M., Steiniger, S.C., Kreuter, J., Zimmer, A., 2005a. Cationic lipid-protamine-DNA (LPD) complexes for delivery of antisense c-myc oligonucleotides. Eur. J. Pharm. Biopharm. 60, 287–294.

Junghans, M., Loitsch, S.M., Steiniger, S.C.J., Kreuter, J., Zimmer, A., 2005b. Cationic lipid–protamine–DNA (LPD) complexes for delivery of antisense c-myc oligonucleotides. Eur. J. Pharmaceut. Biopharmaceut. 60, 287–294.

Kim, T.W., Kim, Y.J., Chung, H., Kwon, I.C., Sung, H.C., Jeong, S.Y., 2002. The role of non-ionic surfactants on cationic lipid mediated gene transfer. J. Control. Rel. 82, 455–465.

Law, S.-L., Chuang, T.-C., Kao, M.-C., Lin, Y.-S., Huang, K.-J., 2006. Gene transfer mediated by sphingosine/dioleoylphosphatidylethanolamine liposomes in the presence of poloxamer 188. Drug Del. 13, 61–67.

Lee, R.J., Huang, L., 1996. Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. J. Biol. Chem. 271, 8481–8487.

Liu, F., Yang, J., Huang, L., Liu, D., 1996. New cationic lipid formulations for gene transfer. Pharm. Res. 13, 1856–1860.

Lonez, C., Vandenbranden, M., Tuysschaert, J.-M., 2008. Cationic liposomal lipids: from gene carriers to cell signaling. Progr. Lipid Res. 47, 340–347.

Morille, M., Passirani, C., Vonarbourg, A., Clavreul, A., Benoit, J.-P., 2008. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials 29, 3477–3496.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Müller, R.H., Mehnert, W., Lucks, J.S., Schwarz, C., zur Mühlen, A., Weyhers, H., Freitas, C., Rühl, D., 1995. Solid lipid nanoparticles (SLN)—an alternative colloidal carrier system for controlled drug delivery. Eur. J. Pharm. Biopharm. 41, 62–69.

Olbrich, C., Bakowsky, U., Lehr, C.M., Muller, R.H., Kneuer, C., 2001. Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. J. Control. Rel. 77, 345–355.

Romoren, K., Thu, B.J., Evensen, O., 2002. Immersion delivery of plasmid DNA. II. A study of the potentials of a chitosan based delivery system in rainbow trout (*Oncorhynchus mykiss*) fry. J. Control. Rel. 85, 215–225.

Rudolph, C., Schillinger, U., Ortiz, A., Tabatt, K., Plank, C., Muller, R.H., Rosenecker, J., 2004. Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide *in vitro* and *in vivo*. J. Pharm. Res. 21, 1662–1669.

Shidhaye, S.S., Vaidya, R., Sutar, S., Patwardhan, A., Kadam, V.J., 2008. Solid lipid nanoparticles and nanostructured lipid carriers–innovative generations of solid lipid carriers. Curr. Drug Deliv. 5, 324–331.

Sun, X., Hai, L., Wu, Y., Hu, H.Y., Zhang, Z.R., 2005. Targeted gene delivery to hepatoma cells using galactosylated liposome-polycation-DNA complexes (LPD). J. Drug Target. 13, 121–128.

Tabatt, K., Kneuer, C., Sameti, M., Olbrich, C., Muller, R.H., Lehr, C.M., Bakowsky, U., 2004. The impact of nanobiotechnology on the development of new drug delivery systems. J. Control. Rel. 97, 321–332.

Tranchant, T., Besson, P., Hoinard, C., Delarue, J., Antoine, J.M., Couet, C., Gore, J., 1997. Mechanisms and kinetics of a-linolenic acid uptake in Caco-2 clone TC7. Biochim. Biophys. Acta 1345, 151–161.

Trotta, M., Debernardi, F., Caputo, O., 2003. Preparation of solid lipid nanoparticles by a solvent emulsification–diffusion technique. Int. J. Pharm. 257, 153–160.

Vaysse, L., Gregory, L.G., Harbottle, R.P., Perouzel, E., Tolmachov, O., Coutelle, C., 2006. Nuclear-targeted minicircle to enhance gene transfer with non-viral vectors *in vitro* and *in vivo*. J. Gene Med. 8, 754–763.

Ward, C.M., Read, M.L., Seymour, L.W., 2001. Systemic circulation of poly (L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. Blood 97, 2221–2229.

Yuan, H., Chen, J., Du, Y.-Z., Hu, F.-Q., Zeng, S., 2007. Studies on oral absorption of stearic acid SLN by a novel fluorometric method. Colloids Surf. B: Biointerf. 58, 157–164.

Yuan, H., Miao, J., Du, Y.-Z., You, J., Hu, F.-Q., Zeng, S., 2008. Cellular uptake of solid lipid nanoparticles and cytotoxicity of encapsulated paclitaxel in A549 cancer cells. Int. J. Pharm. 348, 137–145.

Ziemienowicz, A., Gorlich, D., Lanka, E., Hohn, B., Rossi, L., 1999. Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. Proc. Natl. Acad. Sci. 96, 3729–3733.