International Journal of Pharmaceutics 425 (2012) 62–72

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Novel biomimetic vectors with endosomal-escape agent enhancing gene transfection efficiency

Xiaoli Sun, Chunxi Liu, Donghua Liu, Peng Li, Na Zhang*

The School of Pharmaceutical Science, Shandong University, 44 Wenhua Xi Road, Ji'nan, Shandong Province 250012, China

ARTICLE INFO

Article history:
Received 24 August 2011
Received in revised form
23 December 2011
Accepted 7 January 2012
Available online 14 January 2012

Keywords:
DOPE-g-PLL-b-PEG
PIC
LPCM
Non-viral gene vector

ABSTRACT

Low cytotoxicity and high transfection efficiency are critical issues in designing current non-viral gene delivery vectors. In the present study, a novel biomimetic lipid-polycation copolymer, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-graft-poly(L-lysine)-block-poly(ethylene glycol) (DOPE-g-PLL-b-PEG) was first synthesized and the potential of this novel hybrid lipid-polycation as efficient gene vector was further evaluated. DOPE-g-PLL-b-PEG and DNA could self-assemble into lipid modified polyion complex micelles (LPCM) through electrostatic interactions. Compared with PEG-b-PLL/DNA polyion complex micelles (PIC), LPCM could protect DNA from plasma, nuclease degradation in vitro and showed lower cytotoxicity to HepG2 and HeLa cells ($P < 0.05$). The results of transfection study in vitro indicated that LPCM exhibited higher gene expression than PIC. Especially, the corresponding LPCM displayed the highest transfection efficiency in HeLa cells ($P < 0.05$) when DOPE grafting ratio reached up to 30%. These results suggested that LPCM could facilitate gene transfer in cultured cells and might alleviate the drawbacks of the conventional cationic vector/DNA complexes. As a novel hybrid lipid-polycation, DOPE-g-PLL-b-PEG was valuable to be evaluated for its further application as gene carrier in vivo.

1. Introduction

Gene therapy, the expression of genetic material with therapeutic activity in cells, holds great potential for the treatment of human diseases, and lots of efforts have been made for developing efficient gene transfection vectors. Different strategies including ultrasound transfection (Delalande et al., 2010), particle bombardment (Uchida et al., 2009) or electroporation-mediated delivery of naked DNA (Marshall et al., 2010) have been used as gene delivery systems. However, their applicability is restricted to specific circumstances. The lack of efficient delivery systems is limiting the full therapeutic potential of nucleic acid technology and represents a challenging hurdle in this field. Modern gene delivery relies on the use of nanocarriers that facilitate the delivery of DNA or RNA to the target intracellular organelles (Won et al., 2011). In recent years, non-viral gene vectors have attracted a lot of attentions. Non-viral gene vectors are potential safer alternatives to viral vectors, and may overcome several problems encountered in viral vector-mediated therapy, including immune responses, limited DNA carrying capacity, recombination and high cost (Canine and Hatefi, 2010; Tros De Ilarduya et al., 2010). Cationic polymers and lipids are the most widely used nano-vectors in non-viral gene delivery.

Cationic lipids demonstrated tremendous potential as safe, efficient and scalable in vitro carriers of nucleic acids. However, the lipoplexes consisting of cationic lipids and nucleic acids have been largely found to be insufficient and cytotoxicity for delivery of nucleic acids in vivo (Dass, 2002; Rao, 2010). Compared with cationic lipids, cationic polymers are one of the most popular non-viral gene vectors due to their excellent ability to compact anionic genes, synthetic controllability, better flexibility achieved simply by varying the chemical composition, molecular weight (Mw), and architecture (linear, randomly branched, dendrimer, block, and graft copolymer) (Kabanov, 1999). Poly(L-lysine) (PLL) is one of the first cationic polymers employed for gene transfer. At physiological pH, the amino groups of PLL are positively charged and interact ionically with the negatively charged DNA which protects the DNA and condenses it into nanoparticles (Wagner et al., 1991). An additional advantage is the biodegradability of PLL due to its peptidic nature. However, PLL has a tendency to aggregate and precipitate depending on the ionic strength, especially has poor gene transfection ability when applied alone or without modifications (Farrell et al., 2007).

To overcome the defects of PLL, poly(ethylene glycol) (PEG), a widely used polymer with good biocompatibility, non-toxicity as well as ease of excretion from living organisms, is commonly

* Corresponding author. Tel.: +86 531 88382015; fax: +86 531 88382548.
E-mail address: zhangnancy9@sdu.edu.cn (N. Zhang).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2012.01.010

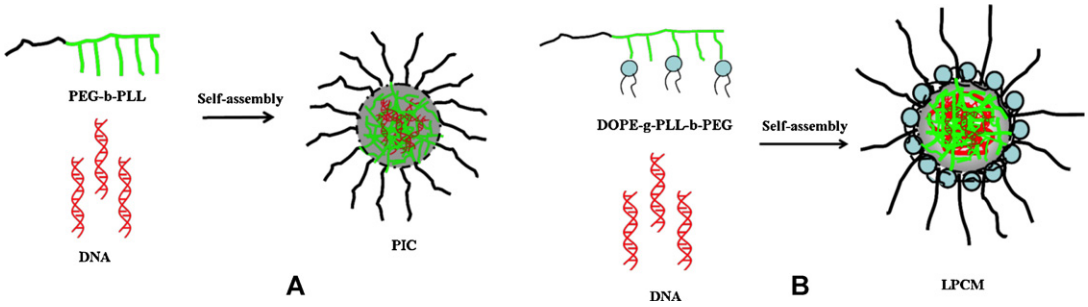

Fig. 1. Formation of polyion complex micelles (PIC) and lipid modified polyion complex micelles (LPCM).

used to modify the PLL based vectors. The groups of Kataoka and Kabanov (Kabanov and Kabanov, 1995; Kataoka et al., 1996) have used block copolymers of polyethylene glycol-b-polylysine (PEG-b-PLL) to form polyion complex micelles (PIC, see Fig. 1(A)), where PLL electrostatically interacts with DNA to form the hydrophobic core, surrounded by the hydrophilic PEG corona. Due to the highly dense PEG shell surrounding the PIC core, the PIC exhibited excellent stability in aqueous media, low cytotoxicity, high tolerability toward nuclease degradation, compared to conventional polyplex and lipoplex systems (Oishi et al., 2007). Although PIC possessed some advantages, the general efficacy and utility have been hampered by lacking endosomal escape, compared to other vectors such as PEI, which can promote its escape to the cytosol from endosomes via the "proton sponge effect". In these cases, PLL conjugation with endosomolytic agents could enhance efficiency of the carrier (Meyer et al., 2008).

Due to significant components of cellular membranes are lipid-derived, one approach of improving the performance of polymer-based non-viral gene vectors is to incorporate hydrophobic components into the vectors. The hydrophobic lipid components that are conjugated to polycations may influence some steps as binding to the cell surface, cellular uptake, escape from the endosomal–lysosomal network, translocation to the cell nucleus, and vector unpacking. The biomimetic vector with hybrid lipid-polycation offers the possibility of enhancing transfection efficiency by facilitating transfer of polyplexes through phospholipid-rich cellular membranes. Hybrid lipid-polycation as gene delivery vector may be divided into as several strategies. The first strategy is mixed methods: "programmed packaging", such as Leaf et al. prepared cationic lipid–protamine–DNA (LPD), which involves the interaction of plasmid DNA with protamine sulfate, then followed by the addition of DOTAP cationic liposomes (Li and Huang, 1997). The polycation PLL, and PEI was also used to condensed DNA to form core, as multifunctional envelope-type nano device (MEND) (Kogure et al., 2004), which involved three steps, DNA condensation with a polycation PLL, then lipid film hydration for the electrostatic binding of the condensed DNA, lastly sonication to package the condensed DNA with lipids. The second strategy of hybrid lipid-polycation is synthetic new materials combining lipid and polycation. Studies have shown that lipid components can significantly alter some of the physicochemical and biological properties of polycations used in gene delivery (Masotti et al., 2007). Kim developed a water-soluble lipopolymer (WSLP), which was synthesized by conjugating a cholesterol moiety to polyethylenimine (800 Da). WSLP was amphiphilic in nature and had advantages: lipophilic cholesterol that can integrate into low density lipoprotein (LDL) and entered cells via the LDL receptor-mediated endocytosis; hydrophilic amine groups of PEI that can facilitate complex formation with DNA and disruption of endosomes by proton buffering effect (Kim et al., 2009). The third strategy of hybrid lipid-polycation is, the synthetic lipid-polycation followed with lipid material to prepare nanoparticles. For example, a novel micelle-like nanoparticle (MNP) were engineered by condensing plasmid DNA with a chemical conjugate of phospholipid with PEI and then coating the complexes with an envelope of lipid monolayer additionally containing polyethylene glycol–phosphatidyl ethanolamine (PEG–PE), resulting in spherical 'hard-core' nanoparticles loaded with DNA (Ko et al., 2009).

In this present work, a lipid-substituted polymer is to incorporate fusogenic lipid DOPE into the polymer PEG-b-PLL. DOPE is generally considered to be one of the most potent helper lipids in vitro, the fusogenic properties of DOPE facilitate the endosomal escape of lipoplexes through membrane destabilization. DOPE also induces the displacement of the anionic lipids from the cytoplasm-facing monolayer of the endosomal membrane to the opposite direction via a flip-flop mechanism. This novel hybrid

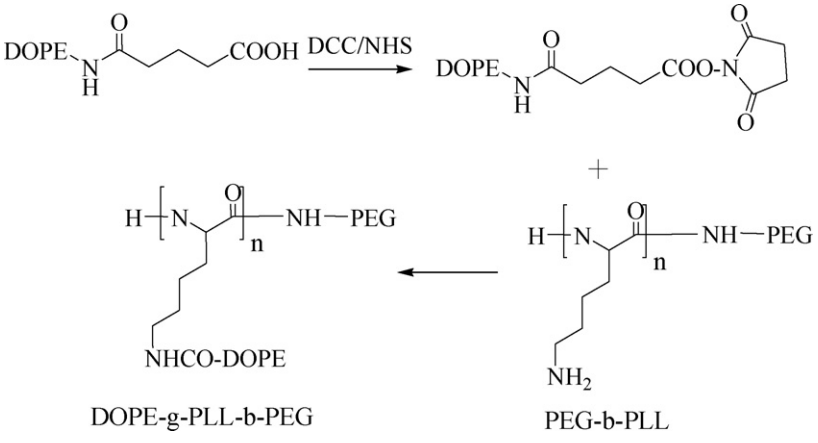

Fig. 2. Synthesis route of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-graft-poly(L-lysine)-block-poly(ethylene glycol) (DOPE-g-PLL-b-PEG).

lipid-polycation copolymer DOPE-g-poly(L-lysine)-block-poly(ethylene glycol) (DOPE-g-PLL-b-PEG) combines the critical properties of a cationic polymer (i.e., DNA condensing ability) with that of a lipid (i.e., membrane fusion capability). The synthetic lipid-polycation DOPE-g-PLL-b-PEG interacted ionically with DNA to form a lipid modified polyion complex micelles (LPCM, see Fig. 2(B)), which have the advantage of cationic polymer and lipids simultaneously: in the core, PLL was applied as DNA binding polycation; DOPE layer formed the coated core, enable intracellular release out of the endosomes; the outer PEG was used as shielding and stabilizing molecule.

In this paper, a systematic and quantitative study of DOPE grafting with PEG-b-PLL is presented. The formation and the physicochemical properties and biological properties of the LPCM is reported, illustrating how the degree of DOPE grafting with PEG-b-PLL affect gene transfection in vitro and cytotoxicity of the transfection vehicle. Furthermore, the DNase I protection studding of the LPCM with time and the plasma stability were investigated as it is crucial for any medical application.

2. Materials and methods

2.1. Materials and cell lines

PEG-b-PLL (MW = 3000–17,000) was a gift kindly provided by School of Chemistry and Chemical Engineering of Shandong University (Jinan, China). DOPE–GA was purchased from Avanti polar lipids, Inc. (Alabaster, AL). 2,4,6-Trinitrobenzenesulfonic acid (TNBs) was purchased from Sigma–Aldrich (China). pEGFP-N_1 was provided by Zhejiang University (China). PicoGreen dsDNA reagent was obtained from Molecular Probes (Invitrogen, USA). Agarose was purchased from BIO-WEST (Spain). Goldview was obtained from Beijing Saibaisheng Biological Engineering Co. (Beijing, China). DNase I enzyme was obtained from Beijing Yinfeng Century Scientific Develop Co., Ltd. (Beijing, China). MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) were purchased from Sigma–Aldrich (China). PEI (25 kDa) was purchased from Sigma–Aldrich (China). HepG2 and HeLa cell line was obtained from American Type Culture Collection (ATCC, USA). All the other chemicals and reagents used were of analytical purity grade or higher, obtained commercially.

2.2. Systhesis of lipid-polycation DOPE-g-PLL-b-PEG

The lipid-polycation DOPE-g-PLL-b-PEG was synthesized by N-acylation of PEG-b-PLL with NHS esters of the lipid, DOPE–GA (Synthesis route see Fig. 2). The DOPE–NHS was synthesized in 40 °C by mixing DOPE–GA, NHS and DCC (1:1.2:1.2 mol) in N,N-dimethylformamide anhydrous. Briefly, the desired amounts of DOPE–GA and NHS were dissolved in 5 mL DMF, then after 4 h, DCC was added dropwise to the mixtures, the reaction was allowed to proceed overnight at 40 °C. The PEG-b-PLL was dissolved in 2 mL DMF, and added into the reaction mixture for 48 h. The solution was put in dialysis bag (molecular weight cutoff 3500 Da, Sigma) and dialyzed against deionized water for 48 h to remove solvent. After filtered, the filtrate was freeze-dried and presented as white powder. DOPE-g-PLL-b-PEG was characterized using IR and ^{1}H NMR spectroscopy.

2.3. Determination of the degree of DOPE modification

The degree of DOPE modification was determined with 2,4,6-trinitro-benzenesulphonic acid (TNBS) assay (Zhang et al., 2008). 1 mL aqueous solution containing free PEG-b-PLL or DOPE-g-PLL-b-PEG was added to 1 mL 0.1 M sodium borate buffer (pH 9.0) and 1 mL TNBS aqueous solution (0.1%), then mixed the solution rapidly. After incubation at 40 °C for 45 min, the reaction solution was stopped by adding 1 mL 0.1 M NaH_2PO_4 containing 1.5 mM Na_2SO_3 and absorption at 420 nm was determined on a UV/VIS spectrometer. TNBS assay was used to determine the free amine content of the lipid-polycation for subsequent studies. The degree of DOPE modification obtained was 16%, 30% and 56%, which was abbreviated as $DOPE_{16}$-g-PLL-b-PEG, $DOPE_{30}$-g-PLL-b-PEG, $DOPE_{56}$-g-PLL-b-PEG in the text below, respectively.

2.4. Formulation of LPCM

The PIC were obtained by means of electrostatic attraction between the anionic plasmid DNA and PEG-b-PLL, while the DOPE-g-PLL-b-PEG and DNA self-assembled into LPCM through electrostatic interactions between DNA and the remaining amino group in PLL. Briefly, about 5 μg of pEGFP was dissolved in 100 μL TE buffer (Tris-EDTA, pH 7.5). Varying concentrations $DOPE_{16}$-g-PLL-b-PEG, $DOPE_{30}$-g-PLL-b-PEG and $DOPE_{56}$-g-PLL-b-PEG, were dissolved in 100 μL of 150 mM NaCl, and then mixed with the DNA solution and incubated at 37 °C for 20 min.

The gels were prepared with 0.8% (w/v) agarose in 20 mL TAE buffer (40 mM Tris, 40 mM Acetic acid, 1 mM EDTA, pH 8.5) containing 2 μL goldview as stains. The resultant complex and control plasmid DNA were applied to gel electrophoresis at a constant 90 V for 20 min. After the electrophoresis, images were obtained using UV transilluminator and Multimage™ Light Cabinet (Alpha Imagers EC, Alpha Innotech Corporation) to show the location of DNA.

2.5. Morphology, particle size and zeta potential

The morphologies of the LPCM and PIC were examined by transmission electronic microscopy (TEM) (JEM-1200EX, Japan), respectively. Samples were prepared by placing a drop of complex onto a copper grid and air-dried, following negative staining with one drop of 2% aqueous solution of sodium phosphotungstate for contrast enhancement. The air-dried samples were then directly examined under the transmission electronic microscopy.

The mean particle size and zeta potential of the LPCM and PIC were analyzed by photon correlation spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, Malvern, England), respectively. All measurements were carried out in triplicates. The average particle size was expressed in volume mean diameter and the reported value was represented as mean ± S.D. (n = 3).

2.6. DNase I protection study

To test whether LPCM protected the loaded plasmid DNA from nucleases digestion, the results of DNase I mediated digestion was performed according to the report (Jeong and Park, 2002). LPCM and PIC were incubated with DNase I (1 U/μL) in DNase I/Mg^{2+} digestion buffer (50 mM, Tris–HCl, pH 7.6, and 10 mM $MgCl_2$), respectively. Naked DNA (1 μg) treated with 1 U/μL DNase I was used as a reference. The UV absorbance change at 260 nm was measured at different intervals by using a UV spectrophotometer.

Table 1
The effect of the formulation parameters on physicochemical characteristics of PIC and LPCM (n = 3).

Samples	Mean diameter (nm) ± SD	Polydispersity index	Zeta potential (mV) ± SD
PIC	118.2 ± 6.9	0.226	+10.4 ± 1.2
$LPCM_{16}$	134.4 ± 5.7	0.385	+19.8 ± 2.4
$LPCM_{30}$	147.4 ± 2.8	0.241	+15.8 ± 5.5
$LPCM_{56}$	188.1 ± 3.9	0.189	+5.8 ± 2.7

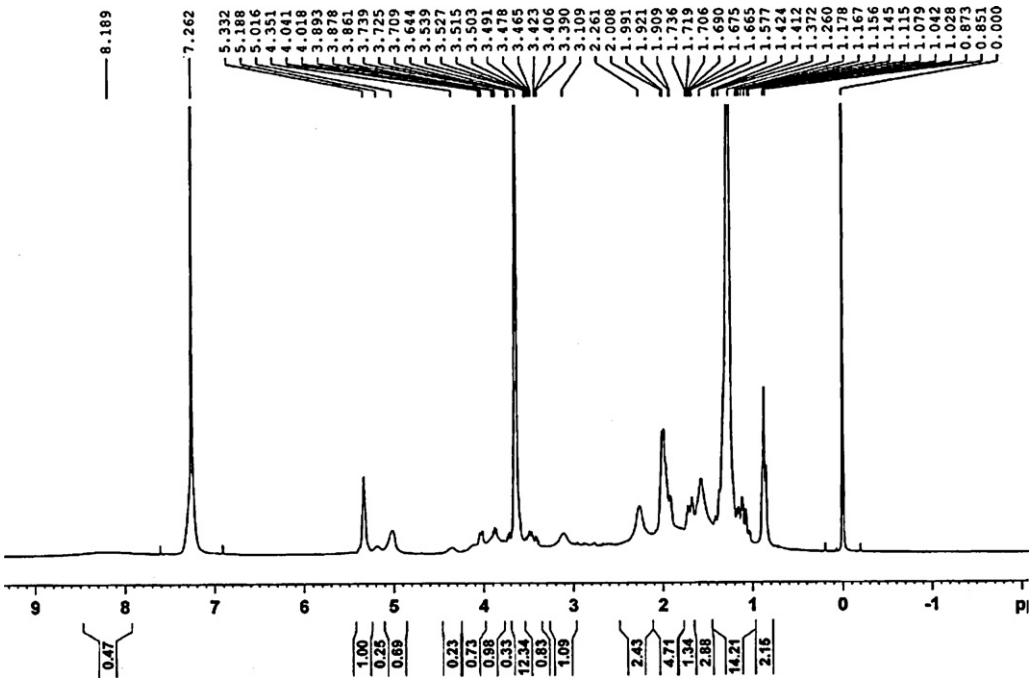

Fig. 3. ^{1}H NMR spectra of DOPE-g-PLL-b-PEG.

2.7. Plasma stability

The stability of LPCM was determined in the presence of human plasma at 37 °C. 20 μL complex were incubated with 20 μL of human plasma at 37 °C for 2 h, and naked DNA incubated with 20 μL of human plasma was used as a control. To asses the integrity of DNA loaded in the complex, heparin solution at final concentration of 1% (w/v) was added into the suspension, and then incubated in shaking water bath (100 rpm) for 3 h at 37 °C. The configuration of plasmid DNA in LPCM after extraction was analyzed by gel electrophoresis with untreated naked DNA as a reference. The samples were applied to a 0.8% (w/v) agarose gel in TAE buffer as described above.

2.8. In vitro cytotoxicity

Cytotoxicity of LPCM was assayed by MTT assay in HepG2 (human liver hepatocellular carcinoma cell line) and HeLa cervical cancer cell lines. The cells were seeded into a 96-well microtiter plates at a density of 8×10^3 cells per well in 0.2 mL of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics in 5% CO_2 incubator at 37 °C over night. After that, the culture medium was replaced by 200 μL fresh serum-free RPMI 1640 medium with LPCM and PIC, respectively. PEI (25 KDa) were used in comparison. After incubating for 48 h, the effect of different treatments on cell viability was assessed by the MTT assay. Typically, 5 mg/mL of MTT in PBS were added to each well reaching a

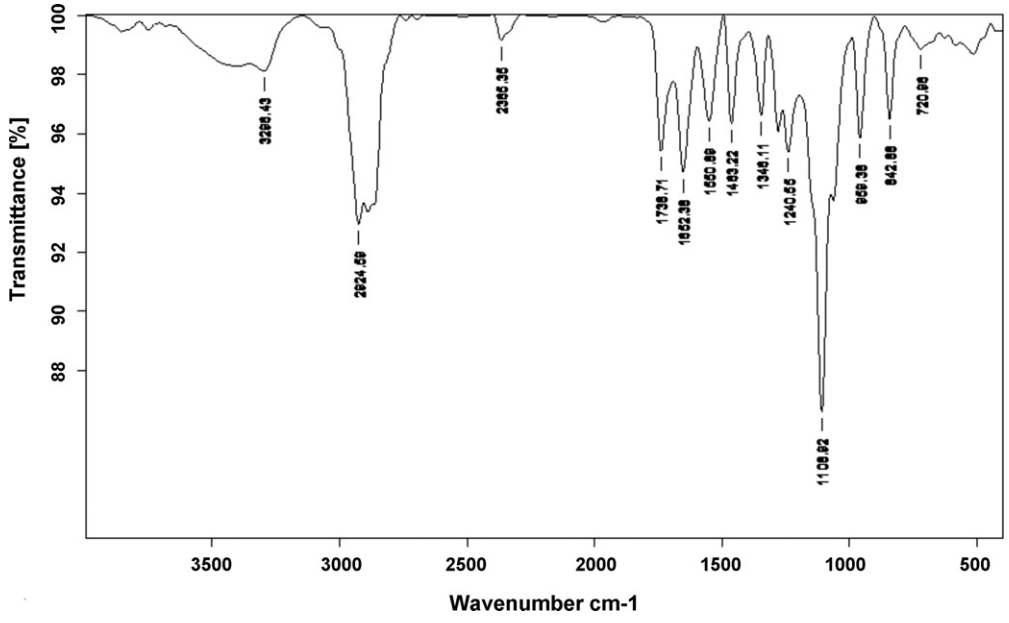

Fig. 4. Infrared spectrogram of DOPE-g-PLL-b-PEG.

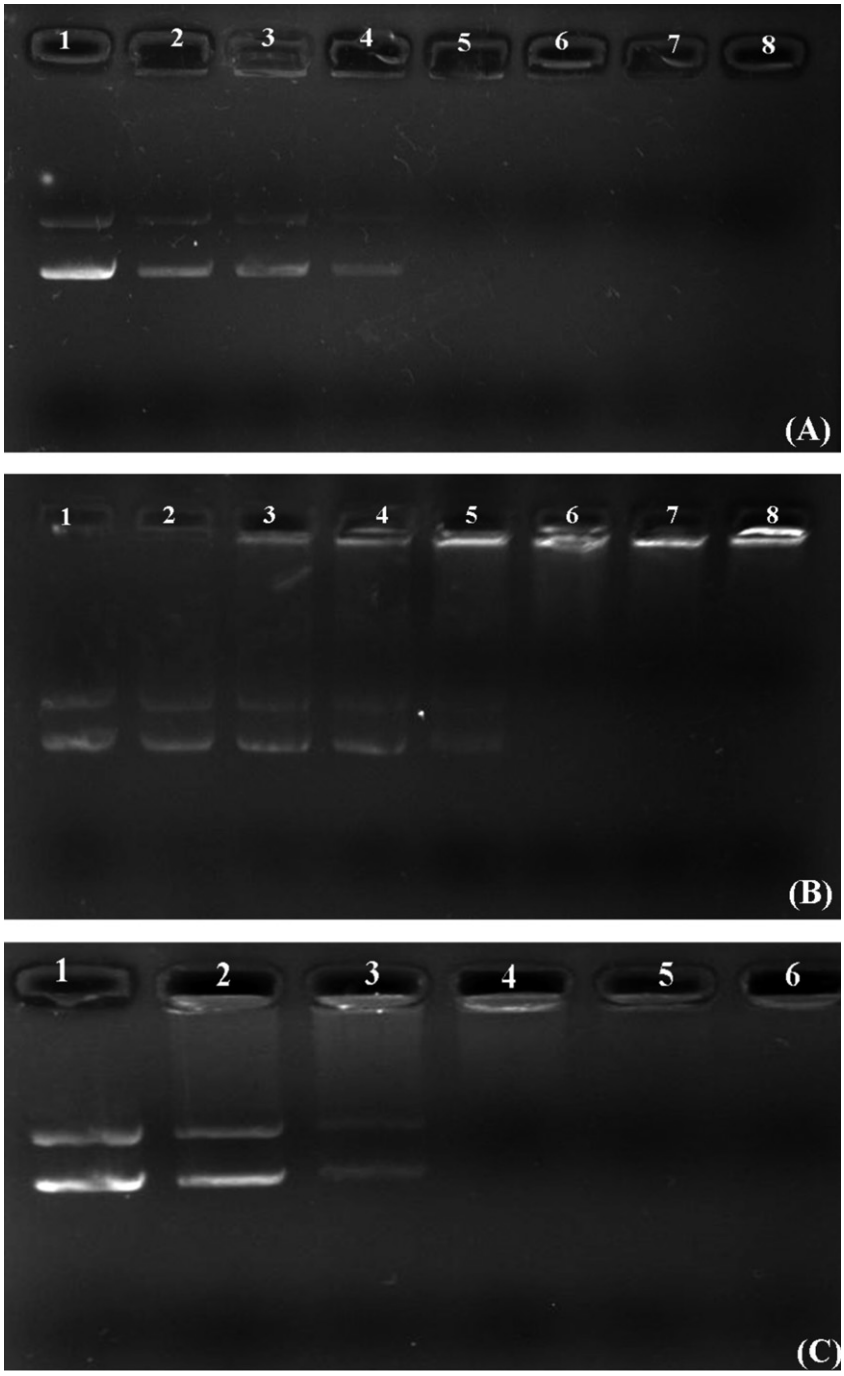

Fig. 5. LPCM formed with different mass ratio. (A) $LPCM_{16}$ formed with different mass ratio. lane 1, naked DNA; lanes 2–8, the ratio of $DOPE_{16}$-g-PLL-b-PEG/DNA was 6:1, 10:1, 12:1, 14:1, 16:1, 20:1 and 25:1 respectively; (B) $LPCM_{30}$ formed with different mass ratio. lane 1, naked DNA; lanes 2–8, the ratio of $DOPE_{30}$-g-PLL-b-PEG/DNA was 1:1, 5:1, 10:1, 15:1, 20:1, 30:1 and 40:1 respectively; (C) $LPCM_{56}$ formed with different mass ratio. lane 1, naked DNA; lanes 2–8, the ratio of $DOPE_{56}$-g-PLL-b-PEG/DNA was 15:1, 20:1, 25:1, 30:1 and 40:1, respectively. DOPE-g-PLL-b-PEG and DNA formed lipid modified polyion complex micelles (LPCM), $LPCM_{16}$, $LPCM_{30}$, $LPCM_{56}$ represented the degree of DOPE modification of the DOPE-g-PLL-b-PEG was 16%, 30% and 56%, respectively.

final concentration of 0.5 mg MTT/mL and incubated for 4 h. Then the supernatants were removed and the formazan crystals were dissolved in 100 μL DMSO. Aliquots were drawn from each well and the absorbance at 570 nm was determined by a microplate reader (Model 680, BIO-RAD, USA). Cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared to control cells were calculated by $(A_{sample}/A_{control}) \times 100$. All treatments were done in quadruplicates and all experiments were repeated in triplicates. A paired *t*-test with $P < 0.05$ was used to establish statistically significant differences between treatments.

2.9. In vitro transfection study

The transfection activity of LPCM was evaluated in HepG2 and HeLa cell lines using plasmid DNA, encoding enhanced green fluorescence protein as reporter gene in the transfection studies. The cells were seeded into 24-well plates at a density of about 1×10^5

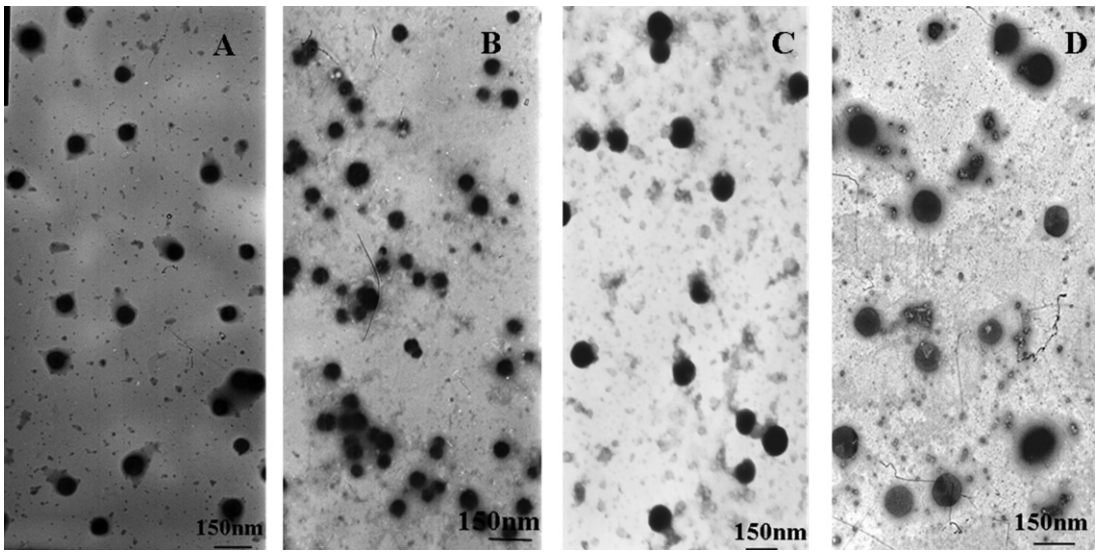

Fig. 6. TEM imaging of complex. (A) PIC (PEG-b-PLL/DNA complex at weight ratio of 6:1); (B) $LPCM_{16}$($DOPE_{16}$-g-PLL-b-PEG/DNA complex at weight ratio of 14:1); (C) $LPCM_{30}$($DOPE_{30}$-g-PLL-b-PEG/DNA complex at weight ratio of 20:1); (D) $LPCM_{56}$ ($DOPE_{56}$-g-PLL-b-PEG/DNA complex at weight ratio of 25:1).

cells per well in 1 mL of RPMI 1640 culture medium with 10% FBS, 48 h prior to transfection. At a confluence level of 70–80%, cells were washed twice with PBS, and, respectively, incubated with 500 μL of media (with or without 10% FBS) containing 2 μg DNA in transfection vectors at 37 °C. PEI/DNA complexes containing 2 μg DNA were used as positive control, while 2 μg plasmid DNA was used as negative control. The cells were incubated with the vectors for 4 h, in the absence of serum medium. The transfection media was then replaced with 1 mL of fresh complete culture media, and the cells were incubated sequentially until 48 h post transfection. Detection of expression of pEGFP-N_1 was carried out using an inverted fluorescent microscope with an attachment for fluorescent observation (OLYMPUS, ZX71, Japan) and the picture was captured using a 4009 objective. After that, all cells were harvested for trypsinization and washed in PBS for three times and cell-associated fluorescence was determined by a FACSC alibur flow cytometer (BD Biosciences) for quantitative study. For each sample, 10,000 events were collected and fluorescence was detected. Signals were amplified in logarithmic mode for fluorescence to determine the positive events by a standard gating technique. The percentage of positive events was calculated as the events within the gate divided by the total number of events, excluding cell debris. Transfection experiments were performed in triplicates.

3. Results

3.1. Design and synthesis of DOPE-g-PLL-b-PEG

The chemical structure of DOPE-g-PLL-b-PEG was confirmed by ^{1}H NMR spectroscopy and IR (Figs. 3 and 4), respectively. IR ν/cm^{-1}: 2924 (—CH_2—, —CH—); 1652 (—HN—CO—); 1738 (—C=O), 1108 (—C—O—C—). ^{1}H NMR spectroscopy was applied in $CDCl_3$, respectively. ^{1}H NMR: δ(3.64), the similar peaks were observed as the spectra of PEG (—CH_2CH_2O—); δ(4.018) and δ(1.26–1.69) showed the spectra of PLL; δ(0.873), δ(5.016) and δ(5.332), the peaks were in concord with DOPE. TNBS reacts with the free amino groups in amino acids and gives absorption at 420 nm. A typical degree of DOPE grafting with PEG-b-PLL using this assay was 16%, 30% and 56%, respectively.

3.2. Formation of LPCM

Gel electrophoresis was performed for assessment of plasmid DNA binding efficiency of LPCM, all lipid-polycations were able to effectively bind and condense the DNA in a concentration-dependent manner (data shown in Fig. 5). DNA was associated with the lipid-polycation to form complex, the mobility of DNA was hindered and it was detained in the well of the agarose gel. When the mass ratio of $DOPE_{16}$-g-PLL-b-PEG/DNA reached 14:1, $DOPE_{30}$-g-PLL-b-PEG/DNA reached 20:1, $DOPE_{56}$-g-PLL-b-PEG/DNA reached 25:1, respectively, almost all DNA was combined with lipid-polycation without free DNA bands in the lane visible. The optimal mass ration of lipid-polycation/DNA was selected for further study.

3.3. Physicochemical characterization

The results of physicochemical characterization of LPCM and PIC were summarized in Table 1. The TEM pictures (Fig. 6) showed that they all had spheroidal shapes. Mean particle sizes of PIC, $DOPE_{16}$-g-PLL-b-PEG/DNA lipid modified polyion complex micelles ($LPCM_{16}$), $DOPE_{30}$-g-PLL-b-PEG/DNA lipid modified polyion complex micelles

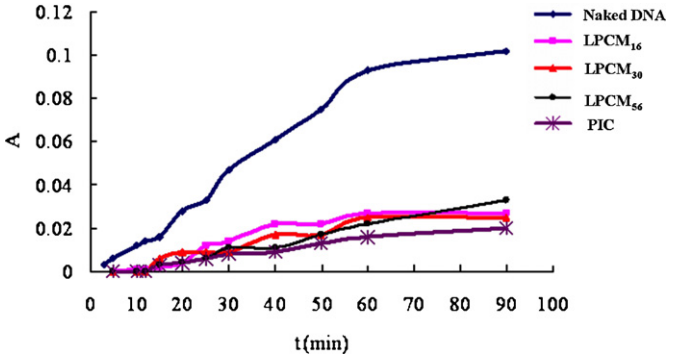

Fig. 7. Variation of UV absorbances at 260 nm against reaction time in DNase I degradation experiments with LPCM. The absorbance value of naked DNA was increasing in 90 min showed that naked DNA was significantly degraded in DNase I, whereas PIC, $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$, the condensed plasmid DNA was efficiently protected from the attack of DNase I.

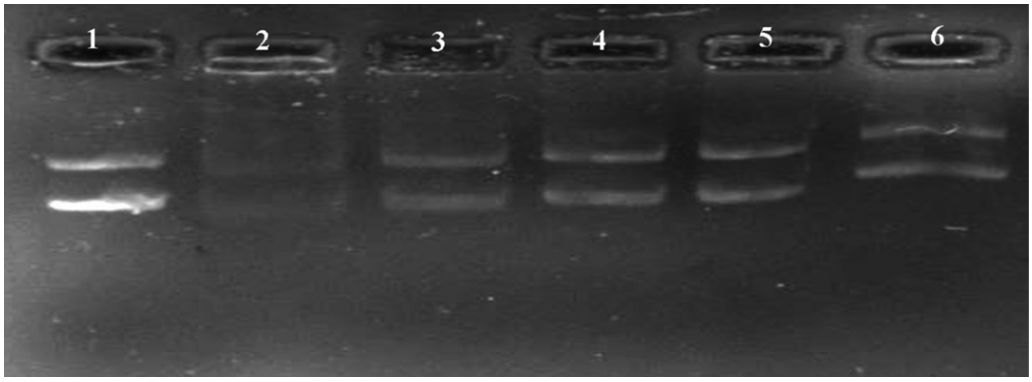

Fig. 8. Stability of LPCM in plasma after incubation in plasma for 2 h. Lane1: naked DNA; lane 2: naked DNA after incubation in plasma for 2 h; lane 3–5: $LPCM_{16}$, $LPCM_{30}$, $LPCM_{56}$; lane 6: PIC. The naked DNA structure had been degraded obviously at the exposure of human plasma for 2 h (lane 2). $LPCM_{16}$, $LPCM_{30}$, $LPCM_{56}$ and PIC remained relatively stable, DNA remain complete structure corresponding to open circular and supercoiled morphology as the naked DNA.

($LPCM_{30}$), and $DOPE_{56}$-g-PLL-b-PEG/DNA lipid modified polyion complex micelles ($LPCM_{56}$) were 108.2, 124.4, 147.4 and 138.1 nm, respectively. Zeta potential of PIC, $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$ were 10.4, 19.8, 15.8 and 7.8 mV, respectively, decreased progressively at the same DNA condensation.

3.4. DNase I protection assay

Protection of plasmid DNA against nuclease attack is one of the crucial factors in designing of an efficient gene delivery in vivo as well as in vitro. Complexation of DNA with cationic polymers is generally considered to prevent the condensed DNA from enzymatic breakdown. Increasing the absorbance value at 260 nm with incubation time indicates the degradation of DNA backbone. The effect of protection to plasmid DNA in all lipopolyplexes from DNase I degradation was examined in the presence of DNase I. Fig. 7 shows naked plasmid DNA was significantly degraded in 5 min, whereas LPCM, the condensed plasmid DNA was efficiently protected from the attack of DNase I.

3.5. Stability study in human plasma

Agarose gel electrophoresis was carried out to investigate whether LPCM could be stable in human plasma. Fig. 8 showed the stabilization of LPCM in plasma. It could be observed that the plasmid DNA had been degraded completely in plasma at the exposure of human plasma for 2 h in lane 2. However, in the presence of human plasma, PIC, $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$ remained relatively stable, DNA still remain complete structure corresponding to open circular and supercoiled morphology as the naked DNA.

3.6. In vitro cytotoxicity evaluation

Some studies showed that cytotoxicity was originated from the structural destabilization of lipid membranes by polycations, but the process of cationic polymer-mediated cytotoxicity is still unclear (Hu et al., 2006). In vitro toxicity of LPCM was evaluated by MTT assay in HeLa and HepG2 cells. The cytotoxicities of PIC and LPCM were evaluated, respectively. PEI/DNA (25 kDa) at the concentration of transfection was used as comparison. As shown in Fig. 9, the cell viabilities in the presence of PIC, $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$ were between 80% and 120%, respectively, exhibited a lower cytotoxicity than PEI/DNA ($P < 0.05$).

3.7. In vitro transfection investigation

The present study intended to evaluate the ability of LPCM to transfer the pEGFP-N_1 to HepG2 and HeLa cells. The fluorescence images in HepG2 and HeLa cells were shown in Fig. 10. The gene transfection efficiency of LPCM was further determined by the flow cytometry quantitively and the data were shown in Fig. 11. Naked DNA without any treatment had some slight green autofluorescence and was used as a negative control. LPCM showed higher transfection efficiency than PIC. Compared with PEI/DNA, after transfection for 48 h, the images indicate that the transfection efficiency of LPCM was similar to that of PEI/DNA complex in HepG2 cells ($P > 0.05$). However, in HeLa cells, $LPCM_{30}$ displayed higher transfection efficiency than PEI/DNA complex ($P < 0.05$).

4. Discussion

Recently, non-viral gene vectors continue to be an attractive alternative to viral gene vectors due to their safety, versatility and ease of preparation and scale-up (Li and Huang, 2006). Investigators in non-viral gene vector development must considered introducing a variety of strategies to overcome barriers for gene delivery, such as plasma membrane, endocytosis and intracellular barriers to traffic as internalization, endosomal escape and nucleus entry (Liu and Zhang, 2009). This has forced investigators to design

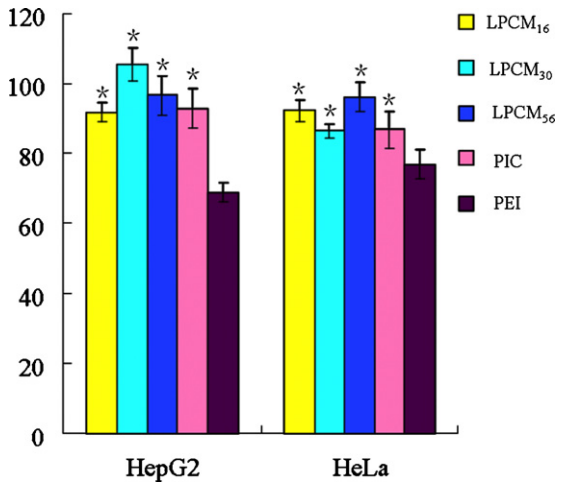

Fig. 9. Cell viability against HeLa and HepG2 cell lines by MTT assay. The cell viabilities of PIC, $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$ were between 80% and 120%, respectively, exhibited a lower cytotoxicity than PEI/DNA ($n = 3$, $^*P < 0.05$ compared with PEI).

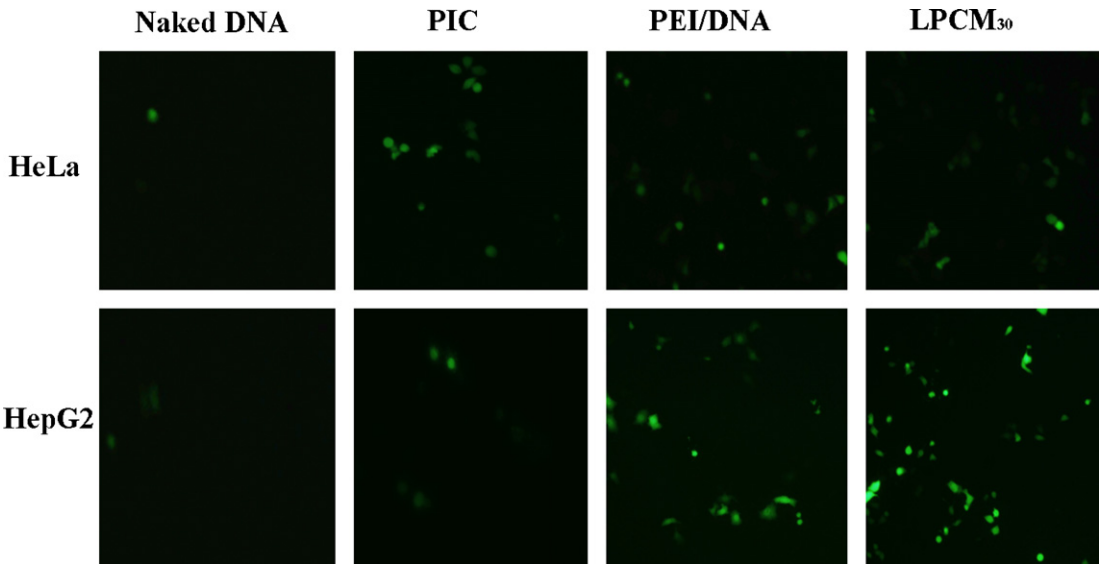

Fig. 10. Gene transfection photo of pEGFP-N_1 in HeLa and HepG2 cells with the naked DNA, PIC, PEI/DNA and $LPCM_{30}$ (200×).

multifunctional non-viral vectors that can overcome the different barriers (Sun and Zhang, 2010).

As the first step to entering cells, the polyplexes must traverse through a hydrophobic lipid-based plasma membrane. The conjugation of hydrophobic lipid segments to the polycations has displayed promising results. Hybrid lipid-polycation might stabilize the complex structure, increase physical encapsulation of genetic materials, promote inversion of complex charge, enhance adsorption to cell membrane, facilitate gene dissociation from polycation carriers in the delivery process (Liu et al., 2010). Low molecular weight oligoethylenimine (OEI) was hydrophobically modified of hexyl acrylate residues, which could enhance the interaction with a cell membrane and promote transfer across the cell membrane barrier by lysis of the endosomes (Philipp et al., 2009). Due to the ability of palmitic acid (PA) to modulate protein–membrane interactions and protein trafficking, PA substitution on low MW PEI was used as an effective approach to increase the lipophilicity of polymers, resulting in better delivery of pDNA into mammalian cells (Remant Bahadur et al., 2011). Abbasi et al. (2008) synthesized lipid-substituted PLL by N-acylation of PLL with NHS esters of fatty acids. The hydrophobic lipid substitution of polycation has been shown to enhance the transfection efficiency in vitro, accordingly be an effective carrier of plasmid DNA.

In our study, the hybrid lipid-polycation was prepared by N-acylation of PEG-b-PLL with NHS esters of the lipid. A commercialization reagent, the lipids DOPE–GA with the carboxyl terminal was used. It has been investigated that DOPE as a colipid promoted hexagonal phase lipid polymorphism that was in favor of membrane fusion, lipid mixing, and boost of transfection efficiency in vitro (Koltover et al., 1998). Cationic polymer PEG-b-PLL was chosen to provide modification sites due to the residual primary amino groups which can be conveniently used for conjugation with lipids. The cationic polymer PLL could readily condense DNA for more efficient uptake. Moreover, the advantages of using PLL as gene vector were its relatively low toxicity and biodegradability. PLL itself was not a good vector for gene transfection, while a lipophilic derivative significantly enhanced the transfection activity (Clements et al., 2007). As presented here, the hybrid lipid-polycation, DOPE-g-PLL-b-PEG combined the advantages of fusogenic lipid, coating material PEG and cationic polymer PLL. The lipid-substituted polymer DOPE-g-PLL-b-PEG self-assembled into micelles in aqueous solution due to the hydrophobic interaction between DOPE molecules which induced the formation of hydrophobic core surrounded by the hydrophilic PLL-b-PEG. When DNA was added, the strong affinity between DNA and cationic PLL-b-PEG segment tend to destroy the micelle structure and to form a lipid modified polyion complex micelles (LPCM), because in this system the electrostatic force between DNA and cationic PLL-b-PEG segment was much stronger than the hydrophobic interaction between DOPE molecules (Sudhir et al., 2008). Eventually, the former DOPE-g-PLL-b-PEG micelle dispersed and was changed to be a structure with a hydrophobic PLL/DNA core adsorpting DOPE on core surface and a hydrophilic PEG shell. Simultaneously, because of the presence of hydrophobic interaction bonds in lipids, the non-polar regions of any of these lipids components will join together to form hydrophobic regions, thus promoting access to be more stable, as biofilm structure for the formation of cell-shielding when phospholipids arranged in the rules. Adsorption of lipids on core surface was driven by electrostatic force which was the main driving force to form the structure of LPCM.

Due to the degree of modification played a major role, a series of modified lipid-polycation with different DOPE degrees were synthesized, the reaction conditions were controlled to substitute <60% amino groups of the PLL, leaving sufficient cations to condense DNA and maintaining the aqueous solubility. The correlation between the pharmaceutical characteristics and the DOPE modification ratio were investigated. In our case, all hybrid lipid-polycation were able to effectively bind and condense DNA to form LPCM at the different lipid-polycation/DNA mass ratio, DOPE grafting led to a reduced tendency of PLL to condense plasmid DNA. The optimal mass ration of PEG-b-PLL was 6:1 (Sun et al., 2010), while the mass ration of three lipid-polycation to DNA reached 14:1, 20:1, 25:1, respectively. Obviously, with the degree of DOPE graft modification increasing, the mass ration of lipid-polycation to DNA increased due to this reduction in charged groups available for binding.

LPCM did result in an improvement in transfection efficiency compared to the PIC or DNA alone in HepG2 and HeLa cells. The impact of DOPE on gene transfection may attribute to the formation of inverted hexagonal phase destabilizing endosomal membrane and facilitating the release of plasmid DNA from lysosomes to protect it from degradation, the endosomolytic character of the DOPE lipid seems to be the reason for improved efficiency (Li et al., 2010). Among polymers with different degree of DOPE graft modification, $LPCM_{30}$ showed the highest transfection efficiency in vitro. PEI

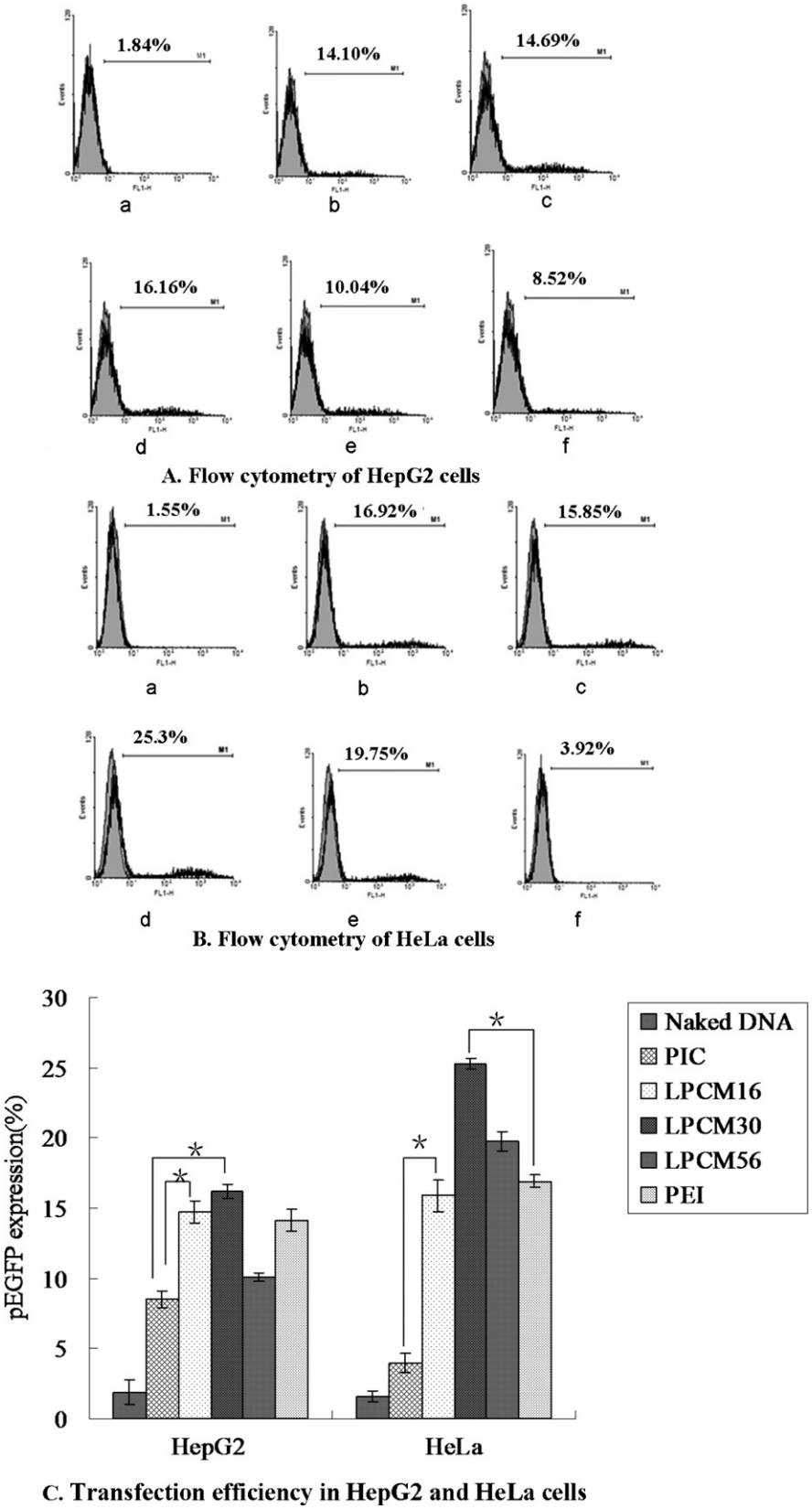

Fig. 11. Flow cytometry of HepG2 (A) and HeLa (B) cells transfected by pEGFP-N_1 with LPCM (*$P < 0.05$), a: naked DNA, b: PEI/DNA, c: $LPCM_{16}$, d: $LPCM_{30}$, e: $LPCM_{56}$, f: PIC. (C): transfection efficiency in HepG2 and HeLa cells. In HepG2 cells, $LPCM_{16}$ and $LPCM_{30}$ showed higher transfection efficiency than PIC, the transfection efficiency of $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$ were similar to PEI/DNA complex. In HeLa cells, $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$ showed higher transfection efficiency than PIC, compared PEI/DNA complex, only $LPCM_{30}$ displayed higher transfection efficiency than PEI/DNA.

(25 kDa) is one of the most efficient nonviral gene transfer agents which currently applied as a golden standard for in vitro transfection. Compared with PEI/DNA complex, the transfection efficiency of $LPCM_{30}$ was similar to that of PEI/DNA complex in HepG2 cells ($P > 0.05$), however, $LPCM_{30}$ displayed a remarkably higher transfection efficiency than PEI/DNA complex in HeLa cells ($P < 0.05$). In our case, when lower degree of DOPE graft with polymer, the ability of DOPE to penetrate the cell membrane is not enough; while higher degree of DOPE graft with polymer, DOPE replaced too many amino groups of PLL, the ability of lipid-polycation binding with DNA was reduced. These studies collectively provided important evidence for critical properties of lipid substituted amphiphilic polymers for effective gene delivery. This might explain why $LPCM_{30}$ displayed the highest transfection efficiency.

On the other hand, minimal or no cytotoxicity of a synthetic gene vector is an important requirement for gene delivery systems. No influences were observed as for the cytotoxicity and the protection against DNase I degradation for the LPCM prepared with different DOPE graft modification with polymer. At the dosage of transfection, no significant cytotoxicities were observed and the cell viabilities of $LPCM_{16}$, $LPCM_{30}$ and $LPCM_{56}$ were all higher than 80% both in HepG2 and HeLa cells. Due to the PEGylation of the polymer, LPCM as well as PIC could provide protection for naked DNA against DNase I degradation. These results indicated the high adaptivity of LPCM, which made them suitable for direct injection into the blood stream or a wound site. In sum, the developed hybrid lipid-polycation DOPE-g-PLL-b-PEG was shown to be beneficial for the development of non-viral gene transfer vectors and may offer an alternative strategy for the future gene therapy.

5. Conclusions

This study provided new insights into the rational design of novel hybrid lipid-polycation, DOPE-g-PLL-b-PEG combined lipid and polycation copolymer, which could be used to package DNA to form a novel LPCM. The results showed that LPCM had small size, good colloidal stability and better protection function from nuclease degradation. The LPCM used here showed excellent transfection efficiency combined with low cytotoxicity in HeLa and HepG2 cells. Furthermore, the result demonstrated that $LPCM_{30}$ displayed the highest transfection efficiency in HeLa cells. All of these characteristics are advantageous compared to plasmid size limitations in viral gene delivery vectors and stability issues in polymer/lipid-based gene delivery systems. Future work will have to address surface functionalization with cell targeting and intracellular routing molecules. The specific modification for cell recognition as the treatment of many diseases has been recognized by many scientific researchers.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

This work was supported by the National Natural Science Foundation (No. 81072585) and the Shandong Province Natural Science Foundation (No. ZR2009CM011).

References

Abbasi, M., Uludag, H., Incani, V., Hsu, C.Y.M., Jeffery, A., 2008. Further investigation of lipid-substituted poly(L-lysine) polymers for transfection of human skin fibroblasts. Biomacromolecules 9, 1618–1630.

Canine, B.F., Hatefi, A., 2010. Development of recombinant cationic polymers for gene therapy research. Adv. Drug Deliv. Rev. 62, 1524–1529.

Clements, B.A., Incani, V., Kucharski, C., Lavasanifar, A., Ritchie, B., Uludag, H., 2007. A comparative evaluation of poly-L-lysine-palmitic acid and lipofectamine 2000 for plasmid delivery to bone marrow stromal cells. Biomaterials 28, 4693–4704.

Dass, C.R., 2002. Immunostimulatory activity of cationic-lipid-nucleic-acid complexes against cancer. J. Cancer Res. Clin. Oncol. 128, 177–181.

Delalande, A., Bureau, M.F., Midoux, P., Bouakaz, A., Pichon, C., 2010. Ultrasound-assisted microbubbles gene transfer in tendons for gene therapy. Ultrasonics 50, 269–272.

Farrell, L.L., Pepin, J., Kucharski, C., Lin, X., Xu, Z., Uludag, H., 2007. A comparison of the effectiveness of cationic polymers poly-L-lysine (PLL) and polyethylenimine (PEI) for non-viral delivery of plasmid DNA to bone marrow stromal cells (BMSC). Eur. J. Pharm. Biopharm. 65, 388–397.

Hu, F.Q., Zhao, M.D., Yuan, H., You, J., Du, Y.Z., Zeng, S., 2006. A novel chitosan oligosaccharide-stearic acid micelles for gene delivery: Properties and in vitro transfection studies. Int. J. Pharm. 315, 158–166.

Jeong, J.H., Park, T.G., 2002. Poly(L-lysine)-g-poly(D,L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. J. Control. Release 82, 159–166.

Kabanov, A.V., 1999. Taking polycation gene delivery systems from in vitro to in vivo. Pharm. Sci. Technol. Today 2, 365–372.

Kabanov, A.V., Kabanov, V.A., 1995. DNA complexes with polycations for the delivery of genetic material into cells. Bioconjug. Chem. 6, 7–20.

Kataoka, K., Togawa, H., Harada, A., 1996. Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. Macromolecules 29, 8556–8557.

Kim, J.M., Lee, M., Kim, K.H., Ha, Y., Choi, J.K., Park, S.R., Park, H., Park, H.C., Ahn, C.H., Kim, S.W., Choi, B.H., 2009. Gene therapy of neural cell injuries in vitro using the hypoxia-inducible gm-csf expression plasmids and water-soluble lipopolymer (WSLP). J. Control. Release 133, 60–67.

Ko, Y.T., Kale, A., Hartner, W.C., Papahadjopoulos-Sternberg, B., Torchilin, V.P., 2009. Self-assembling micelle-like nanoparticles based on phospholipid-polyethyleneimine conjugates for systemic gene delivery. J. Control. Release 133, 132–138.

Kogure, K., Moriguchi, R., Sasaki, K., Ueno, M., Futaki, S., Harashima, H., 2004. Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. J. Control. Release 98, 317–323.

Koltover, I., Salditt, T., Radler, J.O., Safinya, C.R., 1998. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Science 281, 78–81.

Li, J., He, Y.Z., Li, W., Shen, Y.Z., Li, Y.R., Wang, Y.F., 2010. A novel polymer-lipid hybrid nanoparticle for efficient nonviral gene delivery. Acta Pharmacol. Sin. 31, 509–514.

Li, S., Huang, L., 1997. In vivo gene transfer via intravenous administration of cationic lipid–protamine–DNA (LPD) complexes. Gene Ther. 4, 891–900.

Li, S.D., Huang, L., 2006. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther. 13, 1313–1319.

Liu, C., Zhang, N., 2009. Pharmaceutical strategies enhancing cell penetration efficiencies of non-viral gene delivery systems. Curr. Gene Ther. 9, 267–290.

Liu, Z.H., Zhang, Z.Y., Zhou, C.R., Jiao, Y.P., 2010. Hydrophobic modifications of cationic polymers for gene delivery. Prog. Polym. Sci. 35, 1144–1162.

Marshall Jr., W.G., Boone, B.A., Burgos, J.D., Gografe, S.I., Baldwin, M.K., Danielson, M.L., Larson, M.J., Caretto, D.R., Cruz, Y., Ferraro, B., Heller, L.C., Ugen, K.E., Jaroszeski, M.J., Heller, R., 2010. Electroporation-mediated delivery of a naked DNA plasmid expressing VEGF to the porcine heart enhances protein expression. Gene Ther. 17, 419–423.

Masotti, A., Moretti, F., Mancini, F., Russo, G., Di Lauro, N., Checchia, P., Marianecci, C., Carafa, M., Santucci, E., Ortaggi, G., 2007. Physicochemical and biological study of selected hydrophobic polyethylenimine-based polycationic liposomes and their complexes with DNA. Bioorg. Med. Chem. 15, 1504–1515.

Meyer, M., Philipp, A., Oskuee, R., Schmidt, C., Wagner, E., 2008. Breathing life into polycations: Functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables sirna delivery. J. Am. Chem. Soc. 130 (11), 3272–3273.

Oishi, M., Hayashi, H., Itaka, K., Kataoka, K., Nagasaki, Y., 2007. pH-responsive pegylated nanogels as targetable and low invasive endosomolytic agents to induce the enhanced transfection efficiency of nonviral gene vectors. Colloid. Polym. Sci. 285, 1055–1060.

Philipp, A., Zhao, X.B., Tarcha, P., Wagner, E., Zintchenko, A., 2009. Hydrophobically modified oligoethylenimines as highly efficient transfection agents for sirna delivery. Bioconjug. Chem. 20, 2055–2061.

Rao, N.M., 2010. Cationic lipid-mediated nucleic acid delivery: beyond being cationic. Chem. Phys. Lipids 163, 245–252.

Remant Bahadur, K.C., Landry, B., Aliabadi, H.M., Lavasanifar, A., Uludag, H., 2011. Lipid substitution on low molecular weight (0. 6–2.0 kda) polyethylenimine leads to a higher zeta potential of plasmid DNA and enhances transgene expression. Acta Biomater. 7, 2209–2217.

Sudhir, P.R., Shrivas, K., Zhou, Z.C., Wu, H.F., 2008. Single drop microextraction using silver nanoparticles as electrostatic probes for peptide analysis in atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry and comparison with gold electrostatic probes and silver hydrophobic probes. Rapid Commun. Mass Spectrom. 22, 3076–3086.

Sun, X., Zhang, N., 2010. Cationic polymer optimization for efficient gene delivery. Mini Rev. Med. Chem. 10, 108–125.

Sun, X.L., Song, J.B., Liu, D.H., Li, P., Lu, Z.J., Zhang, N., 2010. The studies on peg-b-pll copolymer synthesis and self-assemble nano-gene vector. Chin. J. Biochem. Pharm. 31, 302–305.

Tros De Ilarduya, C., Sun, Y., Duzgunes, N., 2010. Gene delivery by lipoplexes and polyplexes. Eur. J. Pharm. Sci. 40, 159–170.

Uchida, M., Li, X.W., Mertens, P., Alpar, H.O., 2009. Transfection by particle bombardment: delivery of plasmid DNA into mammalian cells using gene gun. Biochim. Biophys. Acta 1790, 754–764.

Wagner, E., Cotten, M., Foisner, R., Birnstiel, M.L., 1991. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc. Natl. Acad. Sci. U.S.A. 88 (10), 4255–4259.

Won, Y.W., Lim, K.S., Kim, Y.H., 2011. Intracellular organelle-targeted non-viral gene delivery systems. J. Control. Release 152, 99–109.

Zhang, N., Chittasupho, C., Duangrat, C., Siahaan, T.J., Berkland, C., 2008. Plga nanoparticle—peptide conjugate effectively targets intercellular cell-adhesion molecule-1. Bioconjug. Chem. 19, 145–152.