Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 350 (2008) 344–350
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Design, synthesis and in vitro evaluation of a novel "stealth" polymeric gene vector

He Guosen [a], Feng Min [b], Luo Xin [b], Subbu Venkatraman [a,*]

[a] School of Materials Science and Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore
[b] The School of Pharmaceutical Sciences, Sun Yat-Sen University, Zhongshan II 74, Guangzhou 510080, PR China

Received 20 June 2007; received in revised form 26 July 2007; accepted 28 August 2007
Available online 6 September 2007

Abstract

We report on the synthesis of a novel gene carrier that has low interaction with serum components, as well as low cytotoxicity. Cationic copolymers composing branched poly(ethylenimine) (PEI) grafted with hydrophilic poly(ethylene glycol) (PEG) and poly(L-lactic acid) (PLLA) or small-molecule oleoyl were synthesized and evaluated as novel gene carriers in this study. The copolymers were complexed with plasmid DNA and the resulting polyplexes were approximately 140 nm in diameter and had a positive surface potential ($\zeta = +13.8$ mV) at the N/P ratio of 10/1. The experiments showed that copolymers with the oleoyl moiety were superior to the other two copolymers (with PLLA), in terms of in vitro gene transfection efficiency. Safety studies using MTT assay indicated much lower cytotoxicity of the oleoyl polyplexes than the pDNA/PEI complexes. The intracellular behavior of the polyplexes was monitored by confocal laser scanning microscopy, and it was found that the polyplexes were internalized into HeLa cells very effectively. At the same time, the plasmid DNA carried by the oleoyl-containing copolymers was found to localize in the nucleus of the recipient cells. One experiment comparing serum-free and serum-containing media indicated that the oleoyl polyplexes may be able to evade the reticulo-endothelial system (RES) better than the PEI–pDNA complex.

Keywords: Gene vector; Cationic copolymer; Intracellular distribution; Polyplex

1. Introduction

Gene therapy is an emerging modality developed to treat both inherited and acquired diseases. Among the available gene carriers, viral vectors appear to be the most effective class, since it is the natural behavior of viruses to infect cells. However, viruses are associated with acute toxicity and immunogenicity; in addition viruses have limited capacity for plasmid incorporation. Non-viral gene vectors are superior to viral vectors with respect to DNA attachment efficiency, non-immunogenicity, easy preparation and low toxicity. Over the past two decades, much effort has been directed to the development of non-viral vectors with different structural features (Niidome and Huang, 2002).

Successful gene therapy depends primarily on the ability of the vector–gene complex (labeled lipoplex for liposome–DNA complexes, and polyplex for a polymer–DNA complex), to navigate through the body to reach the target area. The entry of polyplex into target cells is controlled by several factors including the size of the polyplex (Machy and Leserman, 1983; Zhou and Huang, 1994). A large polyplex cannot be endocytosed easily. There are many unclear interactions among the polyplex and the components of blood-like proteins, platelets and leukocytes; in general, the dose of injected polyplex is cleared from blood quickly. To increase the particle circulation lifetime in the body, "PEG-ellation" using poly(ethyleneglycol) (PEG) is widely used (Gref et al., 1994, 2000; Lim et al., 2000; Keller et al., 2003).

Polycationic polymers like poly(L-lysine) (PLL) bind negatively charged DNA via electrostatic interaction. Such polyplexes show efficient intracellular entry and endosomal release of DNA. It has been reported that higher molecular weight PEI (25 kDa) showed higher efficacy in transfection (Fischer et al., 1999). However, these polymers generated higher cytotoxicity also due to the aggregation of huge clusters of PEI on the cell membrane, which induced necrosis (Fischer et al., 1999). We chose low molecular weight poly(ethylenimine) (PEI)

* Corresponding author.
E-mail address: assubbu@ntu.edu.sg (S. Venkatraman).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.08.054

in our gene vector design because of their biodegradability and exclusion by kidney. An oleoyl chain and poly(L-lactic acid) (PLLA) were chosen to construct the hydrophobic part of the vectors, which were indispensable for micelle or bilayer formation. The vectors were partially grafted with PEG, so that vector clearance from blood would be reduced, and to also provide a "fine-turn" function to keep the appropriate amphiphilic property of the vectors and enabling the polyplex to enter the target cells (Fenske et al., 2002).

PEIs with M_n between 0.8K and 2.0K were employed in the vector synthesis. The synthetic strategy of the oleoyl chain containing vectors is to graft PEI with 2 mol of oleoyl chloride first, since most natural lipids carry two aliphatic chains, then further graft with a given amount of mPEG–NHS (methoxy-terminated PEG at one end and *N*-^{3}hydroxysuccinimide-terminated at the other). To graft PLLA to PEI, we used a method reported by Yoon et al. (2002). The carboxyl group of PLLA was activated by dicyclohexylcarbodiimide (DCC) first, followed by *N*-hydroxysuccinimide (NHS). The activated PLLA was reacted with PEI in the same manner as oleoyl chloride but over a longer time period.

2. Materials and methods

All the syntheses were done under an inert atmosphere. Briefly, 1 mol equiv. of PEI (Aldrich, USA) was dissolved in a certain amount of dry chloroform. To this solution, 2 mol equiv. of oleoyl chloride (Aldrich, USA) were added dropwise over 5 min. The resulting solution was further stirred for 2 h. The attachment of oleoyl segment to PEIs was through a unit reaction, hence yielded 100% 2Oleoyl-PEI intermediates. The given amounts of 2Oleoyl-PEIs and mPEG–NHS (Aktar Inc., USA, 10K) in DMSO reacted overnight with stirring. However, due to the relatively low reactivity of macromolecules, the graft of mPEG to the intermediates was only partially complete. The crude products were purified with dialysis (Medicell International Ltd., UK, MWCO: 12–14,000 Da) against DI water, and obtained after freeze-drying. The molecular weights of the copolymers were determined by ^{1}H NMR spectra (Bruker, 400 MHz, Germany, in $CDCl_3$). Briefly, the characteristic resonances of methyl group of oleoyl, the ethylene groups of PEI and PEG were recorded at δ 0.88, 2.75 and 3.65 ppm, respectively. By comparing the integrations of the three resonances and through normalization, the detailed structural compositions of copolymers 3 and 4 were obtained. The carboxyl groups of different PLLA were activated by an equal molar amount of dicyclohexylcarbodiimide (DCC, Aldrich, USA) first in chloroform, followed by *N*-hydroxysuccinimide (NHS, Aldrich, USA). The activated PLLAs were reacted with respective PEI in the same manner as oleoyl chloride but with longer stirring time. Products with PLLA chain were also purified with dialysis. Similarly, for the copolymers 1 and 2, an identical procedure was employed to process the peaks found at δ 2.75, 3.65 and 5.20 ppm in NMR spectra, attributed to the characteristic resonances of ethylene groups of PEI and PEG, and the methine group of PLLA, respectively. The attachment of PLLA and PEG to PEI resulted in yields of about 60% for both copolymer types.

2.1. Polyplex characterization

Copolymer 3 was dissolved in chloroform and sterilized by passing through a 0.2 μm syringe type filter. A given volume of the solution was transferred into a test tube and the solvent was removed by a purified nitrogen stream and vacuum dry. PBS buffer (pH 7.4, Merck, USA) was added to make the concentration to 1.0 mg/mL, and the solution was sonicated for 10 min at room temperature. The solution of copolymer 4 was prepared similarly. The pGL3-control vector (5.2 kb) coding for the firefly luciferase gene driven by the SV40 promoter and enhancer from Promega was propagated in *E. Coli* and purified by column chromatography. The pDNA was quantified by UV absorbance at 260 nm (UVIpro GAS7300, Uvitrc, UK). Agarose gel analysis (Biocompare Inc., USA) revealed the pDNA to be a mixture of primarily supercoiled plasmid with a small amount of nicked plasmid. To form polyplexes at different N/P ratios, the 250 μg/mL of pDNA in TE buffer (Tris–HCl, EDTA, Sigma, USA) was pipetted into different amount of the copolymer solutions, mixed by pipetting up and down. The polyplexes were incubated for 30 min at room temperature before use. The particle size and size distribution of polyplexes 3 and 4 were determined using photon correlation spectroscopy (PCS) on a Malvern Zetasizer 3000HSA (Malvern Instruments, Malvern, UK). Measurements were performed in triplicate to obtain average volume (D_v) and number diameters (D_n). Particle size distribution was calculated from the ratio of D_v/D_n. The zeta potential of polyplexes 3 and 4 was also obtained from the same equipment using a He–Ne laser beam. All measurements were done at the wavelength of 633 nm at 25 °C with a scattering angle of 90°. Samples were dispersed in 1.0 mM NaCl solution and zeta potentials were calculated from the mean electrophoretic mobility by applying the Smoluchowski equation. The results were the mean of five determinations ± standard deviation.

2.2. Gel electrophoresis

Fifteen microlitres of the polyplexes in different N/P ratios were electrophoresed on 0.8% agarose gel in 1× Tris–boric acid–EDTA (TBE, Merck, USA) buffer at 90 mV until the 1× loading dye ran through 80% of the gel. The gel was stained with 0.5 μg/mL ethidium bromide (Sigma, USA) for 30 min and analyzed using an UV transilluminator.

2.3. Transfection experiments

HeLa cells were maintained in Dulbecco's modified essential medium (DMEM, Sigma, USA) supplemented with 10% defined fetal bovine serum (FBS), and supplemented with 100 mg/mL Penicillin G and 100 mg/mL streptomycin sulfate. Cells were cultured in an incubator at 37 °C and a humidified atmosphere containing 5% CO_2. The cells were plated in cell culture flasks and subcultured before reaching confluency using a 0.1% trypsin solution in EDTA. The culture medium was changed every 2 days. The cells were seeded 24 h prior to transfection into 24-well plates at a density of 5×10^4 cells per well in 2 mL of complete medium. The cells were washed once

with serum-free medium, and different N/P ratio polyplexes containing 2.5 μg of pDNA in 0.5 mL DMEM were added. After incubation for 4 h, the solutions were replaced with FBS containing medium. The cells were washed with PBS 20 h later and lysed with 500 μL of 1× Passive Lysis Buffer with gentle shaking for 15 min. 20 μL of the cell lysate was transferred into a luminometer tube containing 40 μL of LAR II luciferase substrate. The relative luminescence unit (RLU) was measured with a Luminometer (Promega, USA) and the protein assay was performed using Micro BCA Protein Assay Reagent Kit (Pierce, USA).

2.4. Cytotoxicity studies

This experiment was carried out in 96-well plates and 1×10^4 cells per well were seeded. Various concentrations of copolymers and polyplexes, ranging from 5 to 640 μg/mL, were prepared in the DMEM medium. Two hundred microlitres of the sample solution was added to each well and subsequently incubated with the cells for 48 h at 37 °C under 5% CO_2. The medium was then removed and replaced with 20 μL of MTT solution. After further incubated for 4 h, cells were lysed with 10% Triton X-100 plus, 0.1N HCl in anhydrous 2-propanol. The absorbance values were determined at 540 nm using a microplate reader (ELX-800 Bio-Tek, USA). The cell viability (%) was calculated as the absorbance ratio of samples compared with the untreated control (100% cell viability).

2.5. Intracellular transport studies

Copolymer 3 was labeled with fluorescein isothiocyanate (FITC) using the modified method described in the literature previously for labeling of PEI (Godbey et al., 1999). Copolymer 3 at the concentration of 10 mg/mL was mixed with FITC (2 mg/mL) in borate buffer (0.1 M, pH 8.5) and incubated at room temperature on a shaker for 2 h. The unbound FITC was removed by dialysis. pDNA was labeled with rhodamine using the Label IT®TM-Rhodamine Labeling Kit (Mirus Bio Corp., USA). The double-labeled polyplex 3 (N/P = 10) was prepared similarly as described earlier (below Fig. 1). HeLa cells (1×10^5 cells per well) were cultured into a 6-well culture dish pre-loaded with a 22 mm × 22 mm sterile glass coverslip each well for 24 h in 2 mL DMEM medium supplemented with 10% FBS under a humidified atmosphere of 5% CO_2. After the cells were washed twice with PBS solution, 0.2 mL (0.1 mg/mL) of the labeled

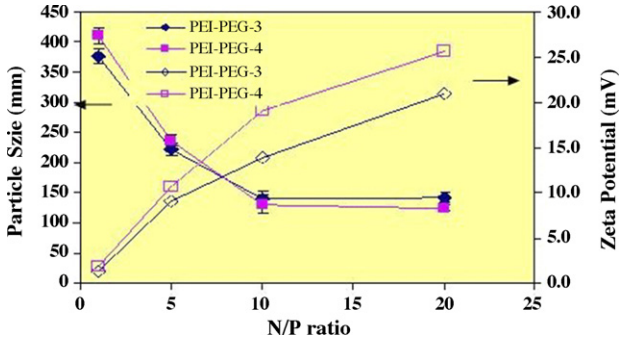

Fig. 1. Size and charge information for polyplexes at different N/P ratios.

polyplexes with 0.8 mL of fresh DMEM medium was added. The cells were then incubated at 37 °C for 2 h, followed by removal of the medium and washing of the cells twice with PBS solution; then fresh DMEM medium containing 10% FBS was added for 4 h more incubation. The cellular entry of labeled polyplex 3 was monitored by confocal laser scanning microscopy (CLSM) (Carl Zeiss, LSM510, Germany). First, the cells treated with samples were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, the coverslips were mounted on glass microscope slides using a drop of antifade solution, diazabicyclooctan (2.5%, w/v). The samples were then observed under CLSM with an argon laser (488 nm excitation) to induce the green fluorescence of FITC and the emission was observed using a band filter (515–565 nm). The He–Ne laser (543 nm) was used to excite the red fluorescence of rhodamine and detected at 575–640 nm wavelengths. The focal plane of each sample was set on the middle of nuclei of the cells. Differential interference contrast images were also obtained.

For CLSM images showing the interacellular distribution of pDNA/Oleoyl–PEI–PEG complexes in a single HeLa cell after transfection, the focal plane of the sample was set on the middle of nuclei. The merged image, the green fluorescent image (515–565 nm filter), the red image (575–640 nm filter) and the differential interference contrast images were all obtained and presented in Figs. 6 and 7, for several HeLa and a single HeLa cell, respectively.

3. Results and discussion

The list of polymers used in this study is shown in Table 1. Higher molecular weights were achieved with copolymers 1 and 2, due mainly to the higher molecular weights of the PLLA

Table 1
Copolymers used in this study, with structural characteristics

No.	Structure[a]	M_w[b]	PEG (%)	PEG/PEI[c]	PEG/PEI[d]
1	2.3PLLA(9.3K)-PEI(0.8K)-2.80 mPEG(10K)	50,000	56.0	35.0	2.80
2	7.0PLLA(5.1K)PEI(2.0K)4.55mPEG(10K)	83,000	54.8	22.8	4.55
3	2Oleoyl-PEI(1.3K)-0.2mPEG(10K)	3,800	52.6	1.5	0.20
4	2Oleoyl-PEI(2.0K)-0.3mPEG(10K)	5,500	54.5	1.5	0.30

[a] Based on calculation from ^{1}H NMR spectra.
[b] Weight percentage.
[c] Weight ratio.
[d] Molar ratio.

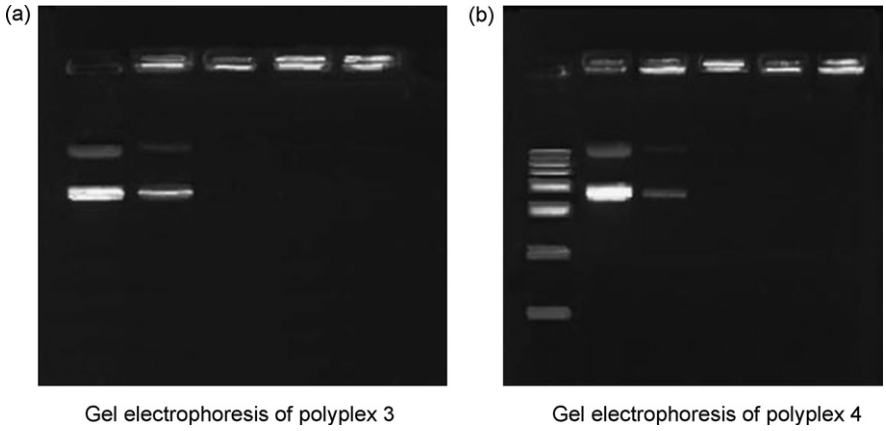

Fig. 2. Gel electrophoresis of pDNA and pDNA-polyplexes. (a) Lines from left to right: 1, pDNA; 2, Polyplex 3, N/P 1:1; 3, Polyplex 3, N/P 5:1; 4, Polyplex 3, N/P 10:1; 5, Polyplex 3, N/P 20:1. (b) Lines from left to right: 1, 1KB step ladder, Promega; 2, pDNA; 3, Polyplex 4, N/P 1:1; 4, Polyplex 4, N/P 5:1; 5, Polyplex 4, N/P 10:1; 6, Polyplex 4, N/P 20:1.

blocks; substitution of oleyl moiety reduced the overall MW substantially. Copolymers 3 and 4 differ in MW by about 30%; the molecular weights of the PEI segments were also different.

Two preliminary experiments to test the transfection efficacy of the four copolymers, at the N/P ratios of 5/1 and 10/1 (ratio of nitrogen-containing groups of the copolymer to phosphate groups of the nucleic acid) on HeLa cells showed that copolymers 3 and 4 were promising, while 1 and 2 were totally inactive. We therefore focused on copolymers 3 and 4 only for a detailed study.

To examine the interaction of plasmid DNA (pDNA) with copolymers 3 and 4, zeta potential and particle size were measured at different N/P ratios. As seen in Fig. 1, the diameters of pDNA/Oleoyl–PEI–mPEG complexes (polyplexes 3 and 4) at various N/P ratios were in the nano-size range and the polydispersity indices were lower than 0.2, suggesting that the range of particle size distribution was narrow in all the cases. The complexation of pDNA with copolymers 3 and 4 led to a significant decrease in pDNA size, resulting in complexes with sizes 10^4 to 10^6 times smaller than that of naked pDNA. Increasing the N/P ratio from 1 to 20 resulted in a decrease in the observed particle size from approximately 400 nm to 120–140 nm, whereas decreasing the N/P ratio from 20 to 1 reduced the positive surface charge of particles from high positive surface charge to near neutral. At the low N/P ratio of 1, the polyplexes 3 and 4 were quite stable and there was no significant difference in the average particle size measured after storage for one month at 4 °C (data not shown). This stability is probably due to the presence of PEG that formed a hydrophilic shell providing steric shielding to the PEI moiety, thus preventing particle aggregation. Compared to the use of PEI alone or the PEI-DNA polyplexes, copolymers 3 and 4 are more stable carriers. At N/P ratios in the range of 5–20, the zeta potential of polyplexes 3 and 4 remained net positive. The positive charge on the complex surface could facilitate adherence to the negatively charged cell membrane, and thus could induce and increase cellular uptake (Duncan et al., 1979).

The formation of polyplexes 3 and 4 at various N/P ratios was investigated by gel retardation assay as shown in Fig. 2. With increase in N/P ratio, pDNA disappeared gradually. It can be found that when N/P ratio ≥5, the pDNA did not migrate at all, suggesting complete complexation inside the polyplexes. The electrostatic adsorption of pDNA on copolymers largely depends on the nitrogen-containing groups of the copolymers. This result is in good agreement with the zeta potential measurement data. At low N/P ratio of 1, although a migrated band of pDNA was observed, zeta potential of these complexes measured was close to neutral. This may indicate coverage of the surface of the complexes with PEG.

The in vitro transfection experiment on HeLa cells in the serum-free medium using different N/P ratios of copolymers 3 and 4 suggested that the optimal N/P ratio is 10/1 for both copolymers. As illustrated by Fig. 3, polyplexes at low N/P ratio

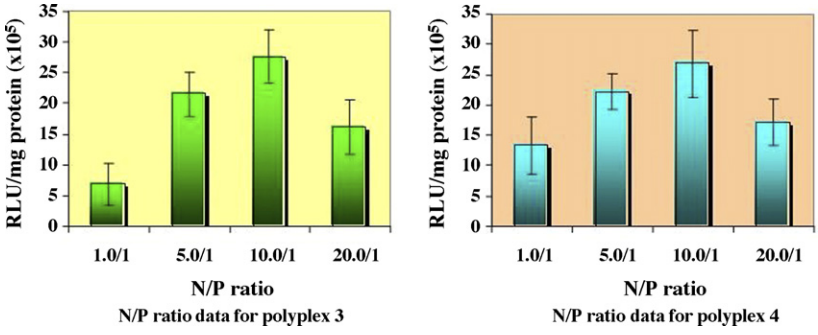

Fig. 3. Charge ratio effect on transfection, for copolymers 3 and 4.

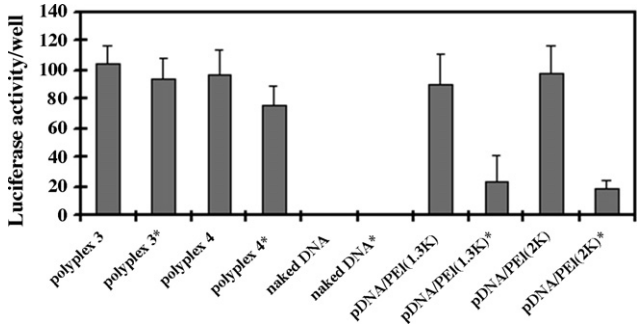

Fig. 4. Transfection rates for copolymer polyplexes 3, 4 and PEI–pDNA complexes in serum-free and serum-containing media (marked with *).

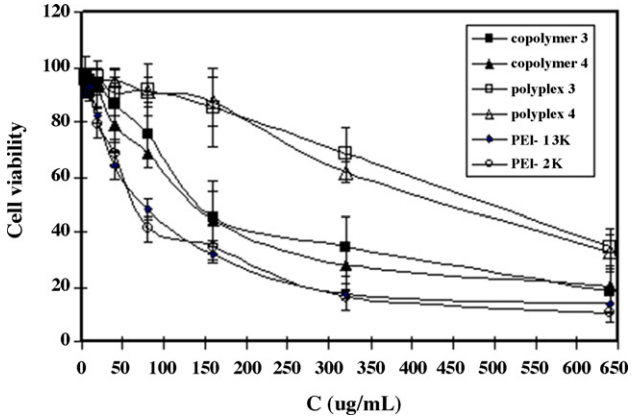

Fig. 5. Cytotoxicity results, expressed as cell viability percent, as a function of polyplex or polymer concentration.

of 1/1 did show some activity compared to the totally inactive naked DNA even though the pDNA was only partially trapped in the vectors (Fig. 2a and b, lines 2 and 3, respectively), suggesting that the copolyplexes were better even at 1/1 ratio. At higher N/P ratios (>15), the transfection efficiencies were lower than those from N/P of 5 and 10, possibly due to the tighter attachment of pDNA to the vectors. Higher positive charge of the vectors may help the entry of polyplexes to cells through electrostatic interaction, since the cell surface is negatively charged, but these vectors may find it more difficult to release the negatively charged pDNA. Intermediate N/P ratios in the range of 5–10 were ideal, with the latter ratio reaching the optimal balance of cell entry and pDNA release.

It is interesting to investigate the blood ingredients-vector interaction to predict the particle lifetime in vivo. Fig. 4 summarizes the transfection results of copolymers 3 and 4, and their precursors, PEI1.3K and PEI2.0K, in the media with and without serum.

Surprisingly, in serum-free medium, both the PEIs produced transfection results comparable with that of copolymers 3 and 4. It appears that under these conditions, the positive charges of PEIs play a dominant role in transfection, as the negatively charged pDNA was totally inactive. However, with 10% serum in the medium, the un-grafted PEIs' transfection efficiency decreased abruptly, while copolymers 3 and 4 showed only a 10–20% decrease. This comparison clearly indicates the opsonization inhibiting property of PEG. The percentage of PEG within the vectors may also be an important factor (Fenske et al., 2002), with the higher PEG content lowering the vector's efficacy. Interestingly, the percentage of PEG in copolymers 1–4 falls into the narrow range 53–56%, so the lower transfection efficiency for copolymers 1 and 2 is not attributable to the PEG content. Other structural parameters shown Table 1 may explain the higher efficiency of copolymers 3 and 4. We believe that the PEG/PEI ratio may be an important contributing factor. A low value for this ratio implies that there is sufficient available PEI to complex well with the pDNA; a high value indicates the converse. Thus for copolymers 1 and 2, the PEG tends to shield the PEI segments, preventing full complexation with pDNA.

Cytotoxicity is an important issue in gene carrier evaluation. Copolymers 3 and 4 and their corresponding polyplexes were tested by MTT assay as shown in Fig. 5. The viability of HeLa cells decreased sharply with the increasing concentration of PEI 1.3K and 2.0K and the average cell viability at the concentration of 80 μg/mL was less than 50%. However, copolymers 3 and 4 showed lower cytotoxicity at the same concentration (86% and 79% cell viability for copolymers 3 and 4, respectively). The average cell viability of the polyplexes at the concentration of 80 μg/mL of the vector was over 90%. From these results, the copolymers and the polyplexes appeared to be less toxic to cells than the PEI. The PEG connected to the copolymer is thought to inhibit its interaction with cellular components and thus reduce the cytotoxicity of the copolymers and polyplexes.

In order to understand the possible mechanism of the transport of pDNA by the copolymers into the cell, we labeled, respectively, the pDNA carrying the firefly luciferase reporter gene (pGL3-Control), with the fluorescent dye rhodamine and the copolymer 3 with FITC before mixing them to form the polyplex. The double-labeled polyplex was added to the HeLa cells in culture and monitored using the Confocal Laser Scanning Microscope (CLSM) with an argon ion laser (488 nm) and a He–Ne laser (543 nm). The fluorescence signal emitted from FITC-labeled copolymer was green and emission from the rhodamine-labeled pDNA was red. The overall emission from the polyplex 3 was yellow (Fig. 6). As can be seen from (a) to (c) that the yellow, green and red fluorescence signals were visible in most of the cells. The fluorescence of FITC and rhodamine co-localized in cells suggested that the polyplex 3 was taken up by most HeLa cells and they possess a very high affinity to the cells. There were also some separate red fluorescent signals observed suggesting that pDNA was detached from the carriers successfully in cells.

The detailed localization of the labeled polyplex 3 in a single HeLa cell was also observed using CLSM as shown in Fig. 7. The particles were internalized and located in the cytoplasm and surrounding the nucleus region of the target cells as shown by the yellow fluorescence signal. The red fluorescence dissociating from the green fluorescence signals in Fig. 7a clearly showed the detachment of pDNA from the vector. The signal emitted from the nucleus region was strong red and yellow (Fig. 7a), indicating that pDNA had been transported into nucleus successfully. The yellow fluorescence shown in the nucleus may suggest that part of the pDNA was also transported into nucleus in the

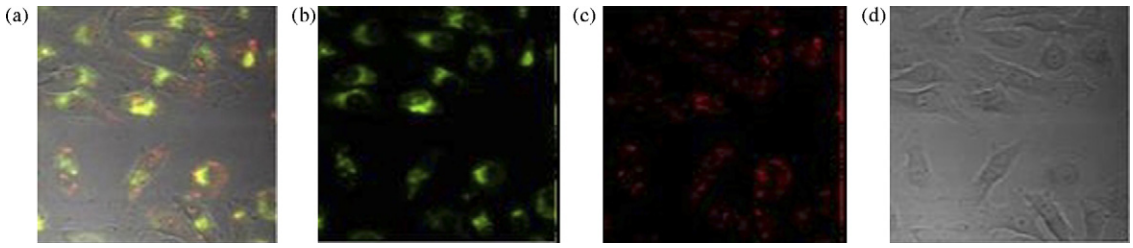

Fig. 6. CLSM images of polyplex 3 in HeLa cells: (a) merged image; (b) green fluorescence at 515–565 nm indicating the localization of FITC-labeled Oleoyl–PEI–PEG copolymer; (c) red fluorescence at 575–640 nm due to rhodamine-labeled pDNA and (d) differential interference contrast image.

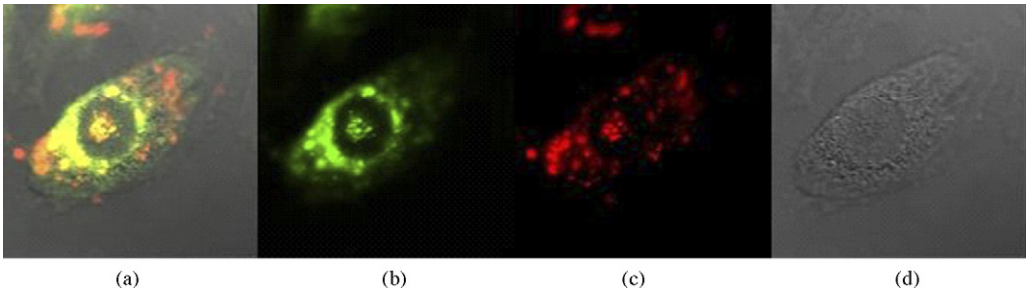

Fig. 7. CLSM images showing the interacellular distribution of pDNA/Oleoyl-PEI–PEG complexes in a single HeLa cell after transfection. The focal plane of the sample was set on the middle of nuclei: (a) the merged CLSM image; (b) green fluorescence indicating the localization of FITC-labeled Oleoyl–PEI–PEG copolymer; (c) red fluorescence, due to rhodamine-labeled pDNA and (d) differential interference contrast image.

form of polyplex. Considering the fact that the complex particles (∼140 nm) had a substantially larger size than the nuclear pore (∼25 nm) (Escriou et al., 2003), it is reasonable to assume that the polyplexes may not passively diffuse into nucleus but enter actively, which in turn suggests that the copolymers were able to protect their payloads on the transport through the extra cellular to the nuclear environment. The discrete green patches shown in Fig. 7b indicated that the polyplexes entered into cells not by diffusion, but they either had been enclosed in vesicles or had formed aggregates in cytoplasm. It may imply that the polyplexes, after attaching onto the cell membrane, were endocytosed and internalized with a number of the polyplexes in each endosome, and this resulted in the aggregated signals from the cytoplasmic region of the cell. These results were consistent with those obtained by Godbey et al. (1999). The endosome's pH is around 5 and is lower than the physiological pH of 7.4. When the pH changes from 7 to 5, PEI, a "proton sponge", will protonate and increase its hydrodynamic volume up to 20–45% (Suh et al., 1994). We speculate that the protonation process causes the detachment of the negatively charged DNA from the complexes and at the same time the excess protons will also trigger the passive influx of chloride ions into the endosome, resulting in osmotic bursting of the endosomes (Kircheis et al., 2001). The pDNA, upon releasing from the complexes, will no longer be enclosed in the endosomes and therefore, is available for transport into the nucleus. However, we have no evidence on how the exogenous DNA was transported into the nucleus.

The photos of a detailed CLSM investigation on a single HeLa cell in slice form focused on the cell from the surface to the bottom confirmed the above observation (figures not shown).

4. Conclusions

In this study, the copolymers composed of a PEI backbone with PEG and Oleoyl/PLLA substructures on both sides were synthesized. It was found that copolymers with partially grafted PEG and oleoyl chains are able to form nanoparticles with pDNA, which have appropriate positively charged surfaces. Copolymers 3 and 4 are proven to possess little cytotoxicity to mammalian cells, low interaction with serum and high transfection efficiency compared to PEI–pDNA complexes in HeLa cells. The low serum interaction suggests less opsonization, and hence potentially less elimination by the RES upon injection into blood. The evidence observed by CLSM showed that the polyplexes internalized and released attached pDNA into HeLa cells very efficiently. Therefore, they have the potential to be biocompatible and efficient gene delivery carriers. These preliminary results suggest that the ideal structural features of the vectors are low PEG content, shorter aliphatic chains and sufficient cations (M_n of PEI > 1K). The copolyplexes are undergoing in vivo testing now in our laboratories.

Acknowledgement

A part of this work was supported by the Sciences Foundation Grant of Guangdong Province (no. 05300666).

References

Duncan, R., Pratten, M.K., Lloyd, J.B., 1979. Mechanism of polycation stimulation of pinocytosis. Biochem. Biophys. Acta 587, 463–475.

Escriou, V., Carriere, M., Scherman, D., Wils, P., 2003. NLS bioconjugates for targeting therapeutic genes to the nucleus. Adv. Drug Deliv. Rev. 55, 295–306.

Fenske, D.B., Maclachlan, I., Cullis, P.R., 2002. Stabilized plasmid-lipid particles: a systemic gene therapy vector. Methods Enzymol. 346, 36–71.

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.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene therapy. Proc. Natl. Acad. Sci. 96, 5177–5181.

Gref, R., Minamitake, Y., Peracchia, M.T., Trubetskoy, V., Torchilin, V., Langer, R., 1994. Biodegradable long-circulating polymeric nanospheres. Science 263, 1600–1603.

Gref, R., Lück, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., 2000. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloid Surf. B Biointerf. 18, 167–186.

Keller, M., Harbottle, R.P., Perouzel, E., Colin, M., Shah, I., Rahim, A., et al., 2003. Nuclear localisation sequence templated nonviral gene delivery vectors: investigation of intracellular trafficking events of LMD and LD vector systems. Chem. Biochem. 4, 286–298.

Kircheis, R., Wightman, L., Wagner, E., 2001. Design and gene delivery activity of modified polyethylenimines. Adv. Drug Deliv. Rev. 53, 341–358.

Lim, D.W., Yeom, Y.I., Park, T.G., 2000. Poly(DMAEMA-NVP)-b-PEG-galactose as gene vector for hepatocytes. Bioconjug. Chem. 11, 688–695.

Machy, P., Leserman, L.D., 1983. Small liposomes are better than large liposomes for specific drug delivery in vitro. Biochim. Biophys. Acta 730, 313–320.

Niidome, T., Huang, L., 2002. Gene therapy progress and prospects: nonviral vectors. Gene Ther. 9, 1647–1652.

Suh, J., Paik, H.J., Hwang, B.K., 1994. Ionization of poly(ethylenimine) and poly(allylamine) at various pH's. Bioorg. Chem. 22, 318–327.

Yoon, J.J., Nam, Y.S., Kim, J.H., Park, T.G., 2002. Surface immobilization of galactose onto aliphatic biodegradable polymers for hepatocyte culture. Biotechnol. Bioeng. 78, 1–10.

Zhou, X., Huang, L., 1994. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochim. Biophys. Acta 1189, 195–203.