International Journal of Pharmaceutics 398 (2010) 237–245

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Poly(α-glutamic acid) combined with polycation as serum-resistant carriers for gene delivery

Cuifeng Wang[a,b], Min Feng[a,b,*], Jingjing Deng[a,b], Yuefang Zhao[a,b], Xin Zeng[a,b], Lina Han[a,b], Shirong Pan[c,a], Chuanbin Wu[a,b]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, University Town, Guangzhou 510006, PR China
[b] Research and Development Center of Pharmaceutics of Guangdong Province, University Town, Guangzhou 510006, PR China
[c] Cardiovascular Laboratory, the First Affiliated Hospital, Sun Yat-sen University, 80 Zhongshan Roas II, Guangzhou 510080, PR China

ARTICLE INFO

Article history:
Received 13 May 2010
Received in revised form 5 July 2010
Accepted 26 July 2010
Available online 3 August 2010

Keywords:
Poly(α-glutamic acid)
DNA terplexes
Serum inhibitory effect

ABSTRACT

The transfection efficiency of cationic polymers decreases dramatically in the presence of serum, which hampers the *in vivo* application of these polymers for gene delivery. Due to its shielding effect of poly(α-glutamic acid) (PGA) from negatively charged serum proteins, it was introduced into DNA polyplexes to overcome the serum inhibitory effect. In the present studies, the transfection efficiency of DNA/PEI/PGA terplex system was compared to PEI 25 kDa and Lipofectamine 2000 in the presence of serum. The successful formation of DNA/PEI/PGA terplexes was confirmed by their near-neutral surface charge. Interaction between components in the terplex system demonstrated that PGA was competing with DNA to combine with PEI. PEI/PGA combined carriers were not cytotoxic at a C/N ratio higher than 0.3. The *in vitro* transfection efficiency of DNA/PEI/PGA terplexes was not significantly different from those of DNA/PEI25 kDa in serum-free medium. Importantly, in serum-containing medium, the DNA terplexes at their optimal C/N ratios maintained the same level of transfection efficiency as that of serum-free medium, even though the transfection efficiency of PEI 25 kDa and Lipofectamine 2000 was significantly decreased under serum-containing conditions. CLSM results confirmed that the cellular import of pDNA delivered by PEI/PGA combined carriers was more efficient than PEI 25 kDa alone under serum-containing conditions. Therefore, PGA could be used as a versatile serum-resistant reagent to overcome the serum inhibitory effect of polycations for gene delivery.

1. Introduction

The success of clinical gene therapy highly depends on the development of safe and efficient gene delivery carriers (Park et al., 2006). Cationic polymers are promising gene transfer carriers since they diminish the risk of pathogenic and immunological complications induced by viral carriers (Pfeifer and Verma, 2001). In many cases these cationic polymeric carriers show high gene transfection efficiency in serum-free medium. However, the *in vivo* efficiency of these carriers is decreased dramatically (Yang and Huang, 1997; Guo and Lee, 2001). One of the main reasons for the inactivation of these carriers is that negatively charged serum proteins bind to the cationic polymers preventing the carriers from binding to the host cells (Haberland et al., 2000).

To overcome serum inhibition of polymer-mediated systemic gene delivery, several strategies have been reported. The most commonly used approach was the chemical modification of polycation using hydrophilic non-ionic poly(ethylene glycol) (PEG) (Kircheis et al., 2001; Shen et al., 2009). Serum was shown to have a relatively weak inhibitory effect on the transfection activity of PEG-modified polyplexes (Tang et al., 2003). It is thought that PEG's bristle like shells surround the DNA polyplexes, reducing non-specific interactions with blood components and surmounts the instability of DNA polyplexes under physiological conditions. However, a high degree of PEGylation was found to significantly reduce the cellular uptake of the polycation and the biological activity of the DNA polyplexes (Petersen et al., 2002).

Poly(α-glutamic acid) (PGA) is a hydrophilic, biodegradable, anionic polymer. Its biological properties such as nontoxicity, biocompatibility, and nonimmunogenicity qualify it for use as a biomaterial in drug delivery and tissue engineering applications (Auzenne et al., 2002; Manocha and Margaritis, 2008). Additionally, PGA is also applied in gene delivery and the results show γ-PGA can improve the intracellular uptake by γ-PGA-specific receptor-mediated energy-dependent process, but α-PGA has no

* Corresponding author at: School of Pharmaceutical Sciences, Sun Yat-sen University, University Town, Guangzhou 510006, PR China. Tel.: +86 20 39943119; fax: +86 20 39943119.
E-mail addresses: fengmin@mail.sysu.edu.cn, minfeng77@yahoo.com.cn (M. Feng).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.07.048

effect (Kurosaki et al., 2009). In our study, we found that PGA was able to significantly improve the transfection efficiency of DNA polyplexes in the presence of serum compared to DNA polyplexes lacking PGA. This is of particular importance for their application as gene carriers *in vivo*.

The purpose of this study was to develop an optimized pDNA/PEI/PGA terplexes which could overcome the serum inhibitory effect on the transfection efficiency of cationic polymeric carriers. Various pDNA/PEI/PGA terplexes with different molecular weights and content of PGA were prepared and characterized. The cytotoxicity of PEI/PGA combined carriers and the interaction between components in their terplexes were also investigated. Transfection efficiencies and cellular entry of pDNA/polycation/PGA terplexes were specifically evaluated by the percentage of cells transfected by the green fluorescent protein (GFP) gene and the fluorescent signal level in host cells under serum-containing conditions.

2. Materials and methods

2.1. Materials

Glutamic acid was obtained from Sinopharm Chemical Reagent Co., Ltd. Polyethylenimine with average molecular weight of 25 kDa was purchased from Sigma–Aldrich. Poly-γ-benzyl-L-glutamate (PBLG) was synthesized by using the molar ratio of monomer to initiator of 100:1 in our laboratory and the detailed synthesis can be found in Pan et al. (2007) and Wen et al. (2009). The pEGFP-C1 plasmid was a gift from West China University of Medical Sciences. EndoFree Plasmid Kit was purchased from Tiangen. The HeLa cell line was obtained from the American Type Culture Collection (ATCC). Lipofectamine 2000 was obtained from the Invitrogen Corporation. Fluorescein isothiocyanate (FITC) and paraformaldehyde were obtained from Sigma. Label IT®TM-Rhodamine Labeling Kit was purchased from Mirus. RPMI-1640 medium, fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Gibco. Agarose was purchased from Biowest. Ethidium bromide was purchased from Invitrogen. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich. All other reagents were analytical grade.

2.2. Synthesis of PGA anionic polymer

The PGA was synthesized via the removal of benzyl groups from poly-γ-benzyl-L-glutamate (PBLG) by hydrogenation as previously described in the literature (Scheme 2) (Lin et al., 2009). In brief, PBLG was first synthesized by the ring-opening polymerization of γ-benzyl-L-glutamic-N-carboxyanhydride (BLG-NCA) using ethylenediamine as the initiator as described in detail in our previous report (Pan et al., 2007; Wen et al., 2009). Then PBLG was dissolved in 40 mL of tetrahydrofuran (THF). 0.8 g/mL of KOH in THF (3× mol ratio to the benzyl groups of PBLG) was added, and the solution was stirred at room temperature for 12, 18 and 24 h. The product PGA was obtained after neutralization by 1.0N HCl, followed by dialysis against water for 48 h using a dialysis bag with a 3.5 kDa molecular weight cut off (MWCO), and finally freeze-drying. The PGA was characterized by ^{1}H NMR, ^{13}C NMR and GPC.

2.3. Preparation of plasmid pEGFP-C1

The plasmid pEGFP-C1 (4.8 kbp) encoding the enhanced green fluorescent protein gene under the control of the human cytomegalovirus (CMV) was replicated in competent high-copy DH5-α *Escherichia coli* strain grown in kanamycin (50 μg/mL) Luria–Bertani medium. Endotoxin-free pDNA was purified by the EndoFree Plasmid Kit according to the manufacturer's protocol. The concentration of pDNA was determined by UV absorbance at 260 nm using an extinction coefficient of 0.02 ($mg^{-1}\,cm^{-1}\,mL$). Additionally, the purity was evaluated by UV spectroscopy (*E* 260/280 nm ratio). pDNA size and homogeneity were assayed by 1.0% agarose gel electrophoresis.

2.4. Preparation of pEGFP-C1/polycation/PGA terplexes

The pDNA/PEI/PGA terplexes were prepared as shown in Scheme 2. Briefly, 50 μg/mL of pEGFP-C1 was mixed with various amounts of 200 μg/mL of PGA in PBS buffer based on C/N ratio (the molar ratio of carboxyl groups of PGA to amino groups of PEI) by gentle vortexing. Then 200 μg/mL of PEI 25 kDa at the optimal N/P ratio of 10 was added to pEGFP-C1/PGA mixture and rapidly mixed by pipetting. The resulting mixture was incubated for 15 min at ambient temperature to yield the pEGFP-C1/PEI/PGA terplexes.

2.5. Size and zeta potential measurements

The particle size and size distribution of pDNA/PEI/PGA terplexes were measured by using a photon correlation spectroscopy (PCS) on a Malvern Zetasizer NS90 (Malvern Instruments, Malvern, UK). The instrument was equipped with a 10-mW helium neon laser producing light at a wavelength of 633 nm and the Malvern PCS version 2.41 software. pDNA complexes were dispersed in 5% glucose solution. Measurements were done in cuvettes at 25 °C with a fixed scattering angle of 90° through a 400 μm pinhole. Each data point is comprised of at least 3 independent experiments.

Zeta potential values were also obtained using a Zetasizer NS90 with a He–Ne laser beam. All measurements were done at a wavelength of 633 nm at 25 °C with a scattering angle of 90°. Samples were dispersed in 10 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.

2.6. Circular dichroism studies

Circular dichroic (CD) spectroscopy was used to investigate the structural interaction between pDNA, PEI and PGA in the terplexes. CD spectra were acquired at 25 °C on a Chirascan™ Circular Dichroism Spectrometer (Applied Photophysics Ltd.). Complexes consisting of PEI and pDNA were formulated at their optimal N/P ratio of 10, and the final pDNA concentration of the complex formulation was 80 μg/mL. To form the terplexes, PEI was added to the pDNA and PGA mixture solution at the N/P ratio of 10 and varying C/N ratios. Samples were equilibrated at 25 °C for 30 min prior to data collection. The equilibration of the samples was indicated by the absence of further changes in the CD signal at longer equilibration time. All CD spectra were taken in a quartz cell of 0.1 cm path length, at wavelengths from 200 to 350 nm. Data points were recorded at every nanometer with a 3.0 s response time.

2.7. Agarose gel electrophoresis experiments

To determine whether the anionic polymer PGA weakens the interaction between DNA and PEI, a series of gel retardation assays was performed by electrophoresis. The pDNA/PEI/PGA terplexes were formulated at the optimal N/P ratio of 10 and varying C/N ratio of PGA to PEI. Each complex sample was loaded into wells of a 1.0% agarose gel prepared in Tris–acetate–EDTA (TAE) buffer containing 0.5 μg/mL ethidium bromide. Samples were subsequently electrophoresed in an electric field of 110 V for 60 min. Bands corresponding to pDNA were visualized on a UV transil-

luminator (Benchtop UV Transilluminator GDS 8000, USA) and photographed.

2.8. Cell viability test

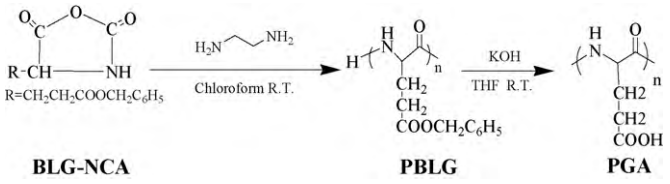
To evaluate whether biocompatible PGA reduces cytotoxicity of DNA/PEI complexes, MTT colorimetric assays were performed. Briefly, HeLa cells were seeded in 96-well plates at a density of 5000 cells/well. After 24 h culture medium was replaced with serial dilutions of samples in full medium and cells were incubated for another 24 h. Then 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in 5 mg/mL in phosphate buffered saline solution was added to each sample. After the samples were incubated for 4 h, the supernatant was aspirated and the formazan crystals were dissolved in 150 μL DMSO. Absorption was measured photometrically at 570 nm with a background correction using a Bio-Tek ELX800 ELISA reader. Values of 8 measurements were normalized to 100% for the control group (exposure to full medium). Cells without addition of MTT were used as a blank for calibration of the spectrophotometer to zero absorbance. The turnover of the substrate relative to control cells was expressed as relative cell viability (%), and was calculated by the $A_{(test)}/A_{(control)} \times 100$ formula.

2.9. In vitro transfection in serum-containing medium

HeLa cells were seeded 24 h prior to transfection into 24-well plates at a density of 1.5×10^4 cells/well in 1.0 mL of culture medium. Prior to the experiment, the cells were rinsed twice with warm phosphate buffered saline (PBS, pH 7.4). 0.1 mL of pDNA/PEI/PGA terplexes and 0.4 mL of serum-free or serum-containing (10%, 20% and 30% FBS) medium were added. The final pDNA concentration was 1.0 μg/well. After transfection for 4 h at 37 °C in 90% humidified atmosphere and 5% CO_2, the cells were rinsed with warm PBS and supplied with 0.4 mL culture medium. After 48 h of incubation allowed for enhanced green fluorescent protein expression, the cells were rinsed twice with PBS. The green fluorescence was observed by fluorescence spectroscopy (Olympus BX51 Fluorescence Spectroscopy, Japan). Subsequently cells were treated with trypsin/EDTA for 2 min, collected by centrifugation, suspended in 0.5 mL PBS and kept on ice until analysis. The percentage of GFP-expressing cells was employed to quantify transfection efficiency via flow cytometry using the Beckman Coulter ESP ELITE System (Beckman Coulter, USA) equipped with an argon laser with an excitation wavelength of 488 nm. The filter setting for emission was 530/30 nm bandpass. Data was acquired in linear mode and visualized in linear mode.

2.10. Confocal laser scanning microscopy study

The cellular entry of Rhodamine-labeled pDNA/FITC-labeled PEI/PGA terplexes under serum-containing conditions was monitored by confocal laser scan microscopy (CLSM) (Zeiss, LSM710). First, the nuclei of the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI). The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After washing with PBS, the coverslips were mounted on glass microscope slides by using a drop of antifade solution, diazabicyclooctan (2.5%, w/v). Then samples were observed under CLSM with a He–Ne laser (568 nm excitation) to induce the red fluorescence of Rhodamine and their emission was observed using a band filter (575–640 nm). The FITC fluorescence was observed using a band filter (515–565 nm) with an argon laser (488 nm excitation) to induce green fluorescence. Blue fluorescence from DAPI was induced by the 350 nm excitation with a UV laser and detected at 430 nm.



Scheme 1. Synthesis of PGA by hydrogenation via removing the benzyl group from PBLG.

3. Results and discussion

3.1. Synthesis and characterization of PGA

PBLG was first synthesized by ring-opening polymerization of BLG-NCA using ethylenediamine as the initiator as previously described (Pan et al., 2007; Wen et al., 2009). PGA having a carboxyl group was obtained by hydrogenation via removing the benzyl group from PBLG using the strong base KOH, as shown in Scheme 1. The complete removal of benzyl groups was confirmed by the absence of benzyl protons at δ5.07 in the ^{1}H NMR spectrum of the obtained PGA polymer (Fig. 1A and B). The PGA peaks in ^{13}C NMR spectrum were quite simple as shown in Fig. 1C. These carbonyl carbon signals were assigned as the side chain δ-carboxylic acid at 178.0 ppm (COOH) and the main-chain amide at 173.5 ppm (C–O). The most upfield resonance peak at 23.5 ppm was assigned to the β carbon of PGA. The resonance peak for the γ carbon of PGA appeared at 31.0 ppm. The resonance peak at 53.5 ppm was attributed to the α carbon of PGA. These five peaks

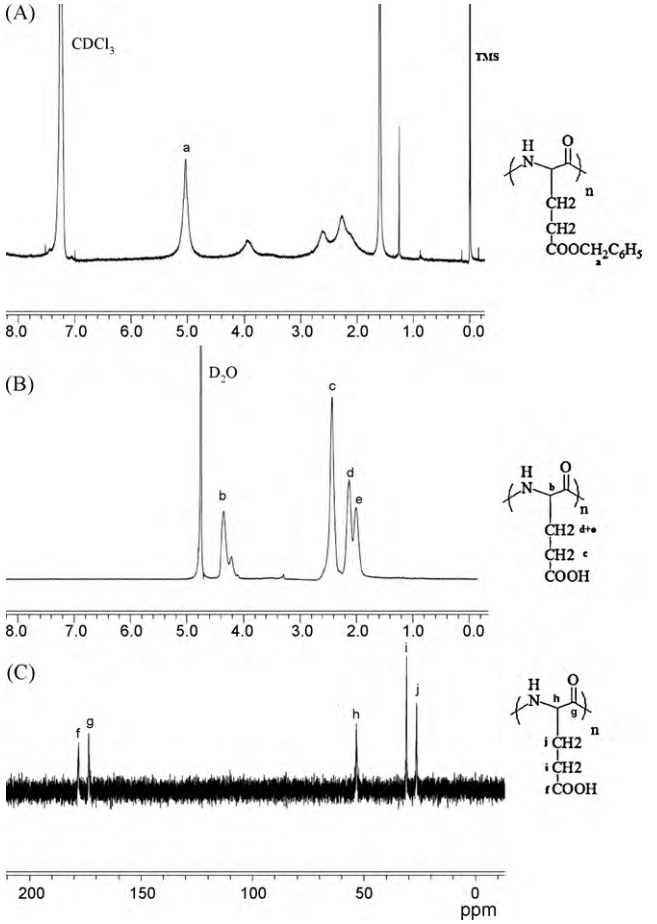


Fig. 1. NMR spectra of PBLG and PGA: (A) ^{1}H NMR spectrum of PBLG; (B) ^{1}H NMR spectrum of PGA; (C) ^{13}C NMR Spectrum of PGA.

Table 1
Characterization of PGA polymers.

Sample	Mw[a] (g/moL)	Mn[a] (g/moL)	Polydispersity[b]
PGA-8K	8329	7516	1.11
PGA-10K	10,060	8481	1.19
PGAI-13K	13,355	10,889	1.23

[a] Mw and Mn as determined by GPC.
[b] PDI as calculated by the Mw divided by the Mn.

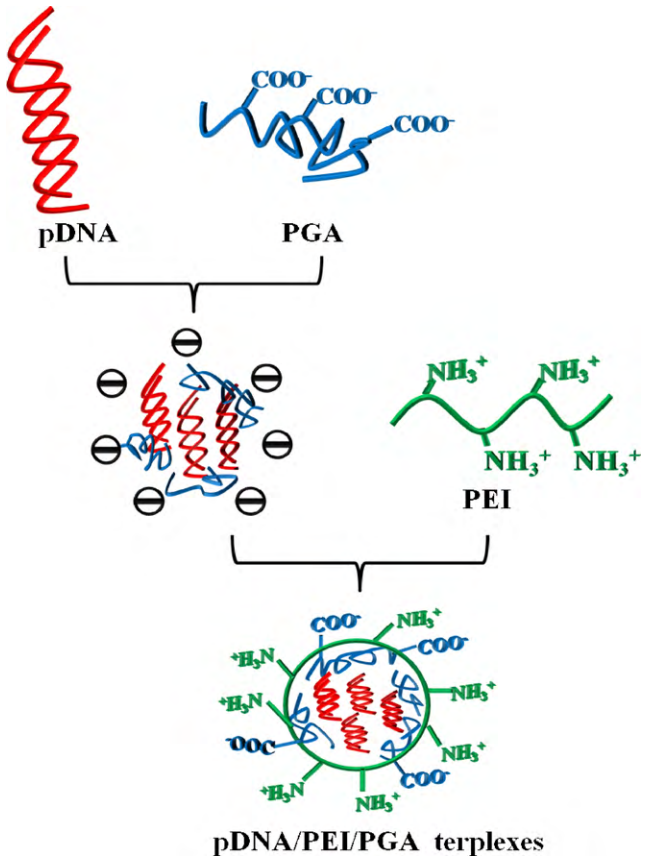
were detected in ^{13}C NMR spectra, indicating that the synthesis of PGA was successful. The molecular weight of PGA was measured by GPC. From GPC analysis, the peak had a unimodal distribution, suggesting that the obtained polymer was free of starting material and the purification was performed sufficiently. Three samples of PGA with the molecular weight in the range of 8329–13355 g/moL were prepared to investigate their serum-resistant properties for gene transfer as shown in Table 1. The polydispersity index (PDI) of the three samples was approximately 1.0. The PDI values suggest that the three samples were nearly a homogeneous polymer with a narrow molecular weight distribution (Rogošić et al., 1996).

3.2. Formation of DNA/PEI/PGA terplexes

PEI 25 kDa is considered the most potent non-viral carrier for gene delivery *in vitro* (Demeneix and Behr, 2005) for its efficient intracellular entry and endosomal release of DNA carried in serum-free medium. However, DNA/PEI 25 kDa polyplexes are inefficient *in vivo* possibly due to the non-specific interactions between the polycation and some negatively charged proteins in blood components (Merdan et al., 2005). In the present study, we found that adding the polyanion PGA to the DNA/PEI 25 kDa polyplexes thereby forming the DNA/PEI/PGA terplexes could overcome the serum inhibitory effect. PGA contains a carboxyl group in the side chain of every repeat unit. These negatively charged carboxyl groups in PGA could interact with the positively charged amines of PEI. In the same way, PEI could also interact with the negatively charged phosphate pDNA backbone via electrostatic interactions. Terplexes using the optimal N/P ratio of 10 and a varying C/N ratio of PGA to PEI, as portrayed in Scheme 2, to optimize their serum-resistant properties. This self-assembled approach to prepare the pDNA/PEI/PGA terplexes circumvented the need for aggressive and reagent-wasteful chemical reactions. Additionally, it was convenient to formulate the different proportions of components in the DNA terplexes.

3.3. The effect of varying the PGA proportion on particle size and surface charge

The slightly positive charge of the DNA complexes is thought to be necessary for the attachment of the complexes to the anionic cell surfaces, facilitating endocytosis as well as low cytotoxicity (Fischer et al., 2003). The surface charge of these terplexes was measured at the N/P ratios of 10 and varying the content of PGA. As indicated in Fig. 2A, the zeta potential of pDNA/PEI polyplexes was approximately 23 mV. After introducing PGA into the complexes, the zeta potential of pDNA/PEI/PGA terplexes was in the range of 12.6 to −17.4 mV. The lower surface charge might be beneficial to balance the cellular uptake and cytotoxic effect. Furthermore, the zeta potential of these DNA terplexes decreased as the C/N ratio of PGA to PEI increased. It also confirmed that pDNA/PEI/PGA terplexes were successfully formed and the formation was simple through complex coacervation induced by charge neutralization. Fig. 2B shows that the particle size of pDNA/PEI/PGA terplexes with varying amount of PGA at the N/P ratio of 10 was approximately 170–800 nm. The size was significantly larger than that of pDNA/PEI polyplexes. These results might be explained that surface charge on the pDNA terplexes was not high enough to stabilize the small particles, resulting in the recombination to bigger particles.



Scheme 2. Formation of self-assemble DNA/PEI/PGA terplexes.

3.4. Interaction of the components in the terplexes

The near-neutral surface charge of DNA terplexes implied that PGA combined with pDNA/PEI polyplexes was via electrostatic interaction with PEI. Here the interaction between pDNA, PEI and PGA was studied using gel retardation assay and circular dichroism (CD) spectroscopy. Fig. 3 shows the retardation assay images of pEGFP-C1/PEI/PGA terplexes at the N/P ratio of 10 and varying C/N ratio of PGA to PEI. When PGA was added to the DNA/PEI polyplexes at the C/N ratio of more than 0.8, a free DNA band was detected. This showed that PGA caused decomposition of the DNA complexes by competitive dissociation of the DNA molecule at the N/P ratio of more than 0.8. However, no DNA band was detected at the C/N ratio of less than 0.8. These results suggested that the PGA combined with PEI by electrostatic interactions as pDNA/PEI polyplexes and pEGFP-C1/PEI/PGA terplexes should be prepared at the N/P ratio of less than 0.8. The released pDNA bands in the gel images seemed brighter in the case of smaller molecular weight PGA-8K and grew fainter as the molecular weight of PGA increased. These results might be due to the fact that the PGA-8K molecules having the smallest bulk volume in the three samples were easier to combine with PEI.

CD spectra are very sensitive to the microenvironment around the probe. Here CD was used to observe the formation of terplexes between pDNA, PEI and PGA, and their conformational changes. Fig. 4 presents the typical CD spectra of the PGA, PEI/PGA complex

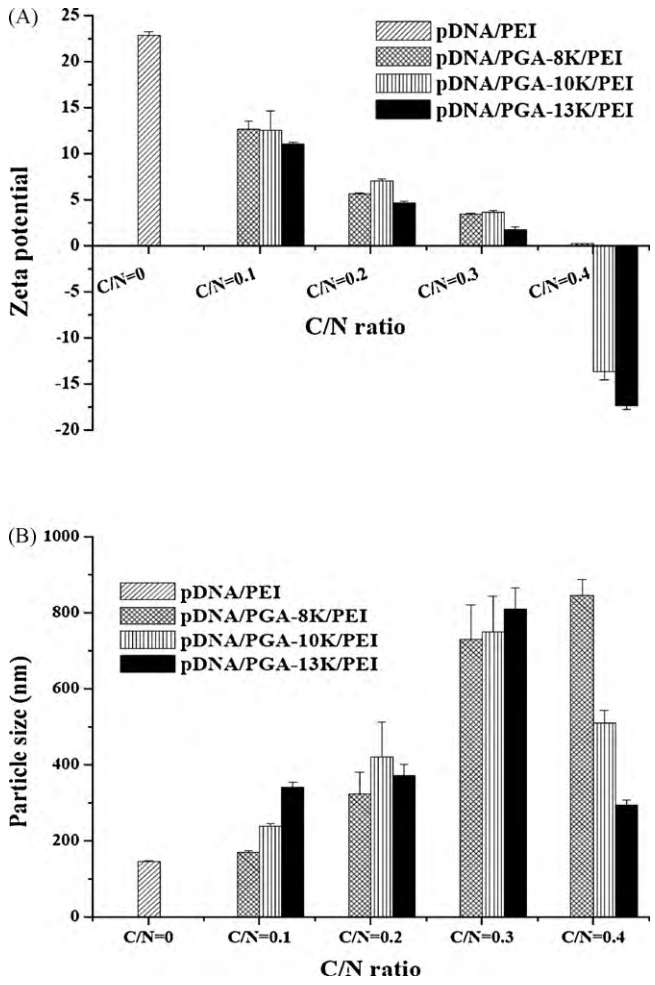


Fig. 2. Zeta potential (A) and particle size (B) of pDNA/PEI/PGA terplexes at N/P 10 and varying C/N ratios of PGA to PEI.

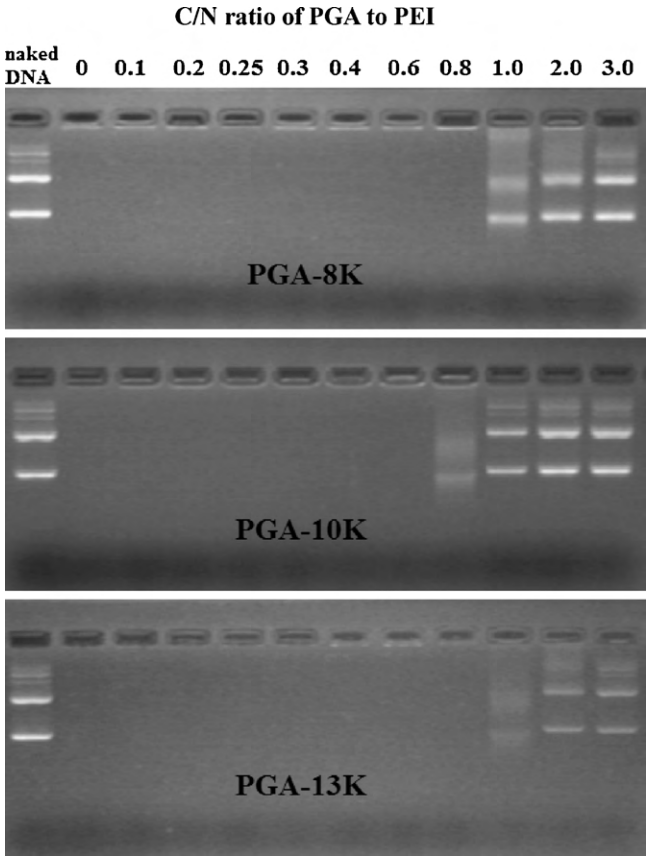


Fig. 3. Agarose gel electrophoresis to assay the formation of pEGFP-C1/PEI/PGA terplexes at the N/P ratio of 10 and varying C/N ratio of PGA to PEI.

and the pEGFP-C1/PEI/PGA terplexes in aqueous solution. CD spectra of PGA (Fig. 4A) in the far-UV region showed a typical inflected curve with a gradual positive band at 210–220 nm and a strong negative band at 198 nm, which was characteristic of a random coil conformation (Wang and Chang, 2003). When PEI 25 kDa was added to PGA-8K at the C/N ratio of 1.0, the CD spectra also showed the same bands at 198 and 218 nm, but a reduction in the molar ellipticity at 198 nm was observed. This indicated that the conformation of the PGA/PEI complex was not different from PGA. The negative band at 198 nm was assigned to the $\pi \rightarrow \pi^*$ transition of peptide (Zubkov et al., 1971). The reduction of this band of PGA/PEI complex suggested that the microenvironment produced by the presence of PEI affected the rotatory strength of $\pi \rightarrow \pi^*$ transition of PGA. This should be due to the electrostatic attraction between anionic PGA and cationic PEI. We further investigated the effect of PGA on the conformation of pDNA in the terplexes. As PGA had no bands in middle-UV region ranging from 230 to 330 nm, the bands of pDNA in the terplexes were analyzed in this region (Fig. 4B). The CD spectrum of naked pDNA presented a typical B-type secondary conformation, which possessed the symmetrical negative band around 245 nm and positive band around 270 nm. Upon pDNA/PGA mixture, the CD spectra did not show any significant change between 240 and 300 nm. However, after PEI 25 kDa was added in the pDNA/PGA mixture at the N/P ratio of 10, the value of the molar ellipticity of the positive band significantly decreased, concomitant with a red shift from 270 to 300 nm and the cross-over around 260 nm shifts more than 30 nm towards longer wavelengths. This suggested that the pDNA was condensed in terplexes, resulting in the collapse of the B-type helical conformation of pDNA and eventually transformating into a C-type geometric conformation with fewer bases per turn (Mahato, 2005). The negative band at 250 nm also shifted in position to higher wavelengths and the molar ellipticity values increased with increasing molecular weight of PGA. This negative band was known to be related to the helicity of DNA (Jaganathan et al., 2008). Thus, it is possible that these CD spectra reflected pDNA condensed by the PEI/PGA-13K combined carrier have stronger helicity with fewer bases per turn than that condensed by the PEI/PGA-8K. We also found that there was a certain trend in changes of CD spectra for different C/N ratio of PGA to PEI. The positive band at 300 nm increased with the increase in C/N ratios in terplexes (Fig. 4C). As the loss of intensity of this pDNA band was attributed to pDNA condensation (Choosakoonkriang et al., 2003), these results might imply that a large amount of PGA loosed DNA condensation in the terplexes. This result was consistent with the result of gel retention assay.

3.5. Cytotoxicity of PEI/PGA complexes

It has been observed that PEI gene transfer activity increased with an increase in molecular weight, whereas their cytotoxicity appeared to increase with increasing polymer size (Kunath et al., 2003). Therefore, PEI 25 kDa showed high gene transfer activity, but also high cytotoxicity that hampered its further biomedical applications. Modified PEI 25 kDa with PEG in order to reduce the PEI's cytotoxicity has been widely investigated. However, these results did not show significant reduction in the cytotoxicity of the PEG grafted PEI under the premise of maintaining the efficient

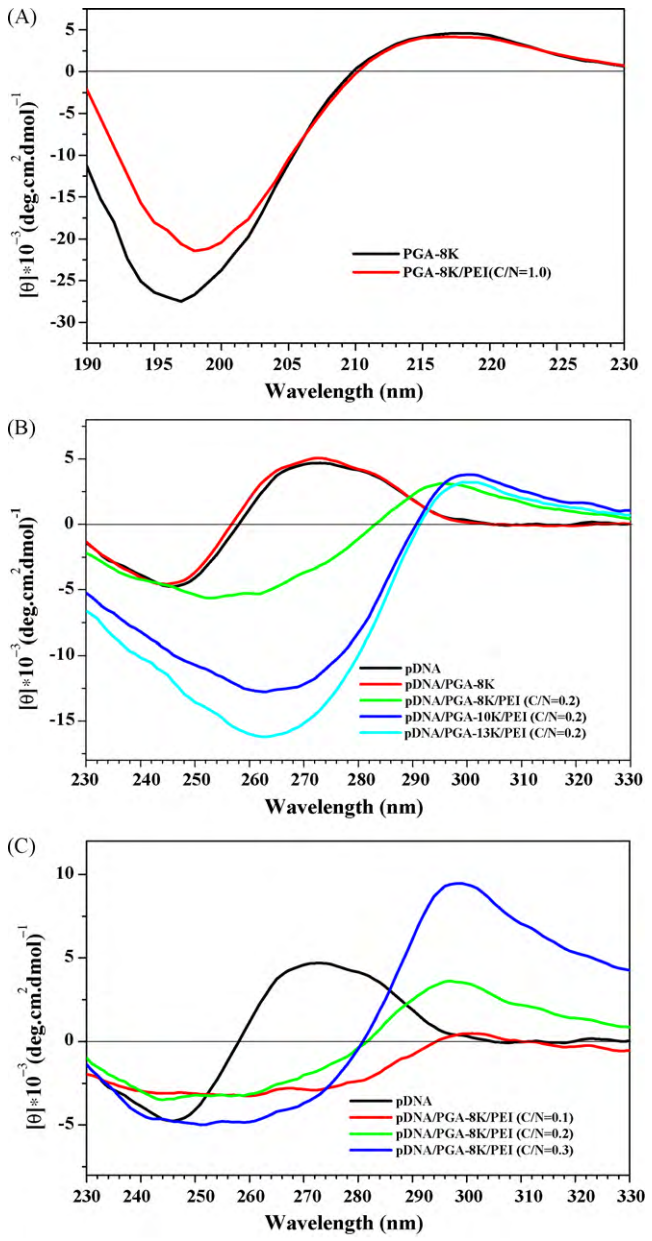


Fig. 4. Circular dichroism spectra of pDNA/PEI/PGA terplexes for the structure of PGA (A); pDNA (80 μg/mL) with PEI at N/P ratio of 10 and PGA with varying molecular weight of at C/N ratio of 0.2 (B); pDNA (80 μg/mL) with PEI at N/P ratio of 10 and PGA at varying C/N ratio (C).

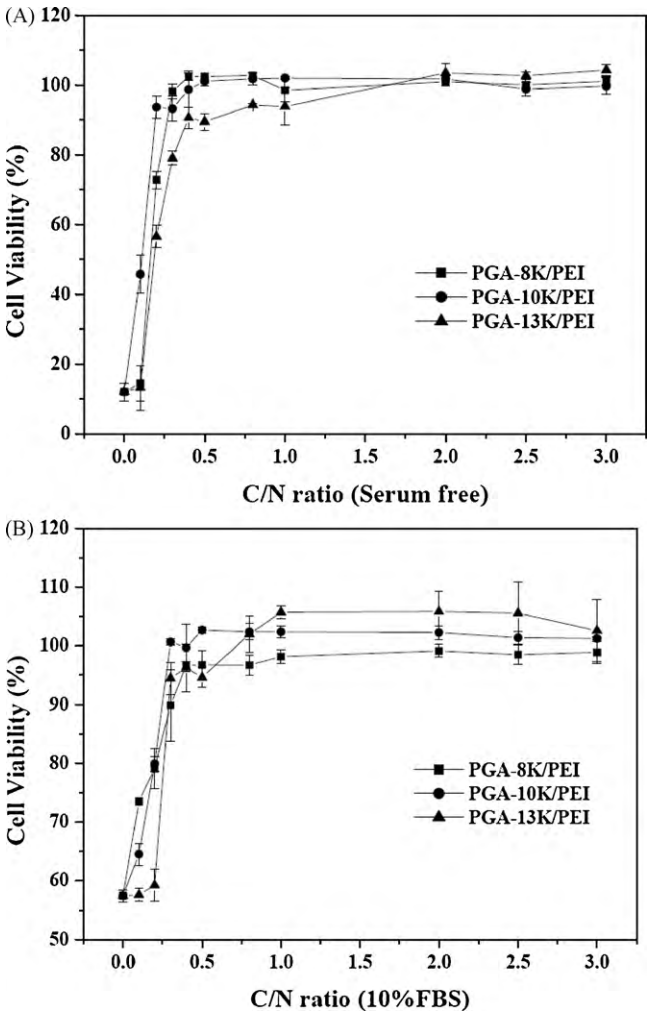


Fig. 5. Cytotoxicity of PEI/PGA combined carriers with the concentration of PEI at 50 μg/mL on HeLa cells (A) in serum-free medium or (B) in serum-containing medium.

transfection (Zhan et al., 2008). Here, biocompatible PGA was introduced into pDNA complexes to reduce their cytotoxicity as well as overcoming the serum inhibitory effect. To determine whether the PEI/PGA combined carriers provide low cytotoxicity, cell viability of the combined carriers at varying C/N ratios of PGA and PEI was assayed by measuring conversion of the MTT tetrazolium compound to formazan. A commercial PEI 25 kDa was used as a positive control to compare the relative toxicity of these combined carriers. Fig. 5 reveals that all of the PEI/PGA combined carriers at the C/N ratio from 0.1 to 3.0 were found to be much less toxic than PEI 25 kDa. The viability of HeLa cells incubated with PEI/PGA-8K combined carriers in the absence of serum was more than 79 ± 3.4% and 90 ± 6.1% at the C/N ratio of 0.20 and 0.30, respectively. In contrast, only 12.5 ± 3.0% of cells incubated with PEI 25 kDa remained viable at the same concentration. Similar results were found in PEI/PGA-10K and PEI/PGA-13K. The cytotoxicity of the three samples with different molecular weights of PGA was not significantly different at all the N/P ratio. The PEI/PGA combined carriers, at the C/N ratio higher than 0.3 and 0.4, incubated with or without serum-containing media, respectively, did not show any cytotoxicity (the cell viability was more than 90%). The surface charge results showed that the zeta potential of PEI/PGA combined carriers at the C/N ratio of 0.3 was neutral. The results demonstrated that the low cytotoxicity of PEI/PGA combined carriers might be attributed to the ionic neutralizing and steric shielding effect of PGA. It also suggested their potential as a gene delivery carrier for further biomedical applications.

3.6. Effect of PGA on the transfection efficiency in serum-containing medium

To investigate whether PGA can overcome the serum inhibitory effect of polycations, the pDNA/PEI/PGA terplexes were assessed for their transfection efficiency in serum-free and serum-containing medium by green fluorescent protein (GFP) assay using pEGFP-C1 at an optimal N/P ratio of 10 and varying C/N (PGA to PEI) ratios (0.1–0.4) as shown in Fig. 6. In parallel, the transfection efficiency of polyplexes consisting of pDNA and PEI 25 kDa was prepared having the same N/P ratio and were compared to pDNA/PEI/PGA terplexes. Commercial Lipofectamine 2000 was also used as a positive control, as it has shown high transfection

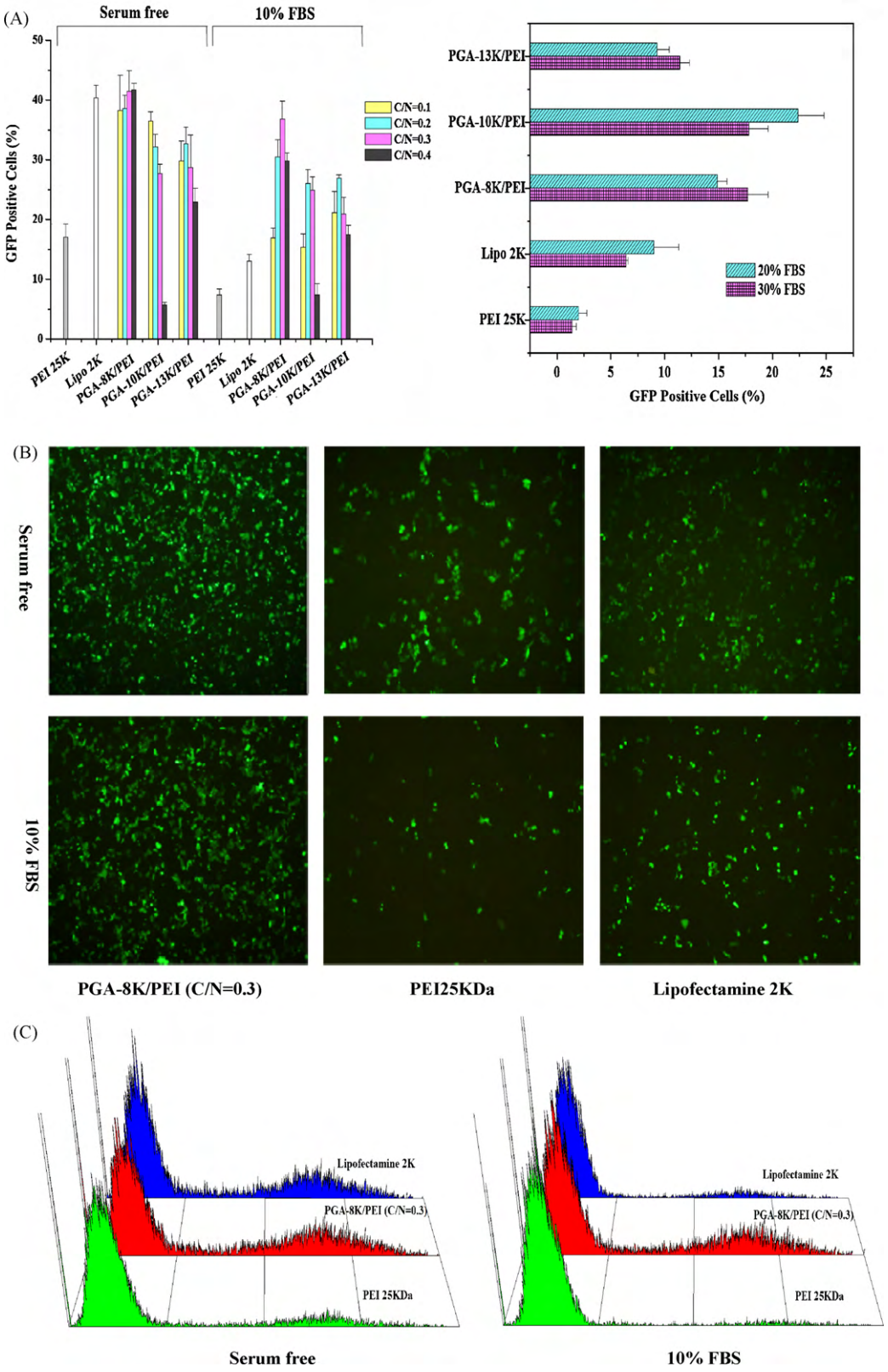


Fig. 6. pEGFP-C1/PEI/PGA terplexes mediated gene delivery in serum-free and serum-containing medium. (A) HeLa cells were transfected with pEGFP-C1/PEI/PGA terplexes at varying C/N ratios (left); HeLa cells were transfected with these terplexes at C/N ratio of 0.2 in 20% and 30% serum-containing medium (right). The percentage of cells transfected was assessed 48 h post-transfection. (B) HeLa cells transfected were analyzed for GFP expression using a fluorescent microscope. (C) FACS analysis.

efficiency. The percentage of cells transfected with pEGFP-C1, as determined by FACS analysis, showed that the transfection efficiency of pDNA mediated by PEI/PGA combined carriers was not significantly different from those by Lipofectamine 2000. Importantly, in 10% FBS containing medium, DNA/PEI/PGA terplexes at their optimal C/N ratios (0.2 and 0.3) showed the same level of transfection efficiency as in serum-free medium. However, the transfection efficiency of PEI 25 kDa and Lipofectamine 2000 sig-

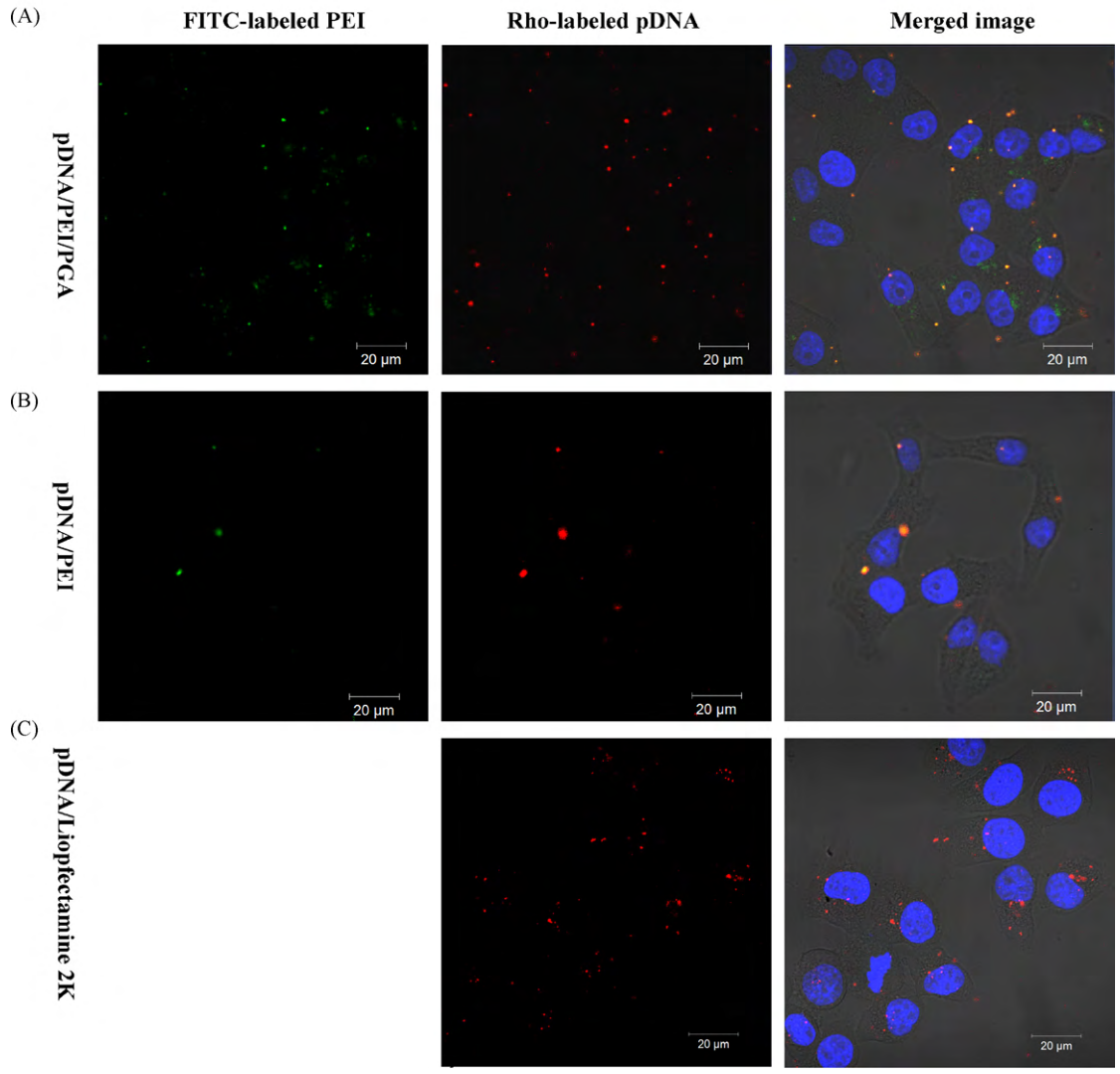


Fig. 7. Confocal micrograph images of intracellular importation under serum-containing condition by (A) Rhodamine-labeled pDNA/FITC-labeled PEI/PGA, (B) Rhodamine-labeled pDNA/FITC-labeled PEI polyplexes, and (C) Rhodamine-labeled pDNA/Lipofectamine 2000 lipoplexes after post-transfection for 3 h. The focal plane of the sample was set on the middle of nuclei. Nuclei were stained blue with DAPI, Rhodamine-pDNA and FITC-PEI was shown as red fluorescence and green fluorescence, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

nificantly decreased under serum-containing conditions. The most effective combined carriers in 10% FBS containing medium was the PEI/PGA-8K combined carrier at the C/N ratios of 0.3, which was 3.4 and 6.0 times more effective than the commercial Lipofectamine 2000 and PEI 25 kDa under the same conditions (Fig. 6A left). Furthermore, all the PEI/PGA samples also showed higher transfection efficiency than those of PEI 25 kDa or Lipofectamine 2000 in 20% and 30% FBS containing medium (Fig. 6A right). This improvement in transfection over PEI 25 kDa or Lipofectamine 2000 under serum-containing conditions could also be seen by comparing the fluorescent micrographs and FACS histogram of GFP-expressing cells produced using PEI/PGA-8K combined carriers with PEI 25 kDa and Lipofectamine 2000, as shown in Fig. 6B and C. In serum-containing medium pEGFP-C1 mediated by PEI/PGA-8K showed a much higher level of GFP fluorescence in HeLa cells than PEI 25 kDa or Lipofectamine 2000. The higher level of fluorescence should be due to the fact that PGA neutralized and shielded highly positively charged PEI and prevented PEI from non-specially interacting with negatively charged serum proteins. Therefore, PGA could be useful as a versatile serum-resistant reagent to overcome the serum inhibitory effect of polycations for gene delivery.

3.7. Effect of PGA on the cellular uptake of pDNA under serum-containing condition

To explore the PEI/PGA combined carrier transporting exogenous DNA into cells under serum-containing conditions, we labeled the pDNA with the fluorescent dye Rhodamine and the PEI/PGA combined carrier with FITC before allowing them to form DNA terplexes. Then the double-labeled DNA terplexes were administrated to the HeLa cells with DAPI stained nuclei in 10% FBS medium culture and monitored by CLSM. In parallel, double-labeled pDNA/PEI binary polyplexes and Rhodamine-labeled pDNA/Lipofectamine 2000 were also prepared under their optimal conditions and were compared to pDNA/PEI/PGA terplexes in terms of cellular entry of pDNA in target cells. The fluorescence signal emitted from the FITC-labeled PEI/PGA combined carrier was green and emission from the Rhodamine-labeled pDNA was red. The overall emission from the pDNA/PEI/PGA terplexes was yellow (the combination of green overlaid with red fluorescence). By 3 h post-transfection under serum-containing conditions, red fluorescence from these three kinds of pDNA complexes and green fluorescence from polyplex carriers were all detected in HeLa cells (Fig. 7). The results suggested that all these pDNA complexes were internalized in

target cells. No significant differences of red fluorescence were found in intracellular uptake between pDNA/PEI/PGA terplexes and pDNA/Lipofectamine 2000 lipoplexes. However, compared to pDNA/PEI binary polyplexes, the pDNA/PEI/PGA terplexes showed much more red and green signals in target cells. The signals indicated that cellular entry of pDNA delivered by PEI/PGA combined carriers was more efficient than pDNA/PEI polyplexes under serum-containing condition. Efficient cell entry of the pDNA/PEI/PGA terplexes might be due to the fact that PGA combined competitively with the pDNA/PEI polyplex and prevented serum proteins from binding the pDNA polyplex. It is thought that PEI non-specially interacting with serum proteins results in aggregation and low transfection efficiency of polyplex. Considering that only different component in the formation between the pDNA/PEI/PGA terplexes and pDNA/PEI binary polyplexes was the PGA, it was reasonable to assume that PGA overcame the serum inhibition of the polycation-mediated gene delivery to some degree.

4. Conclusion

The present work developed a simple method to prepare the pDNA/PEI/PGA terplexes to overcome the serum inhibitory effect of cationic polymers. Biocompatible PGA containing carboxyl groups could self-assemble with positively charged PEI. PEI/PGA combined carriers showed lower cytotoxicity than PEI 25 kDa. The successful formation of pDNA/PEI/PGA terplexes was confirmed by their near-neutral particle surface charge. The interaction between the components in terplexes demonstrated that PGA competed with pDNA to bind to PEI and pDNA/PEI/PGA terplexes could be formed at the C/N ratio of less than 0.8. The transfection efficiency of pDNA/PEI/PGA terplexes was not significantly different from that of DNA/Lipofectamine 2000 in serum-free medium. Importantly, in 10% FBS containing medium, pDNA/PEI/PGA terplexes at their optimal C/N ratios showed the same level of transfection efficiency as in serum-free medium. However, the transfection efficiency of either PEI 25 kDa or Lipofectamine 2000 significantly decreased under serum-containing conditions. CLSM results confirmed that the cellular import of pDNA delivered by PEI/PGA combined carriers was more than that by PEI alone under serum-containing conditions. Therefore, PGA could be used as a versatile serum-resistant reagent to overcome the serum inhibitory effect of polycations for gene delivery.

Acknowledgments

The authors gratefully acknowledge the National Natural Science Foundation of China (Project No. 30600789) and Guangdong Natural Science Fund (Project No. 9151063301000008) for their financial support of this research.

References

Auzenne, E., Donato, N.J., Li, C., Leroux, E., Price, R.E., Farquhar, D., Klostergaard, J., 2002. Superior therapeutic profile of poly-L-glutamic acid–paclitaxel copolymer compared with Taxol in xenogenic compartmental models of human ovarian carcinoma. Clin. Cancer Res. 8, 573–581.

Choosakoonkriang, S., Lobo, B.A., Koe, G.S., Koe, J.G., Middaugh, R., 2003. Biophysical characterization of PEI/DNA complexes. J. Pharm. Sci. 92, 1710–1722.

Demeneix, B., Behr, J.P., 2005. Polyethylenimine (PEI). Adv. Genet. 53, 217–230.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Guo, W., Lee, R.J., 2001. Efficient gene delivery via non-covalent complexes of folic acid and polyethylenimine. J. Controlled Release 77, 131–138.

Haberland, A., Knaus, T., Zaitsev, S.V., Buchberger, B., Lun, A., Haller, H., Böttger, M., 2000. Histone H1-mediated transfection: serum inhibition can be overcome by Ca^{2+} ions. Pharm. Res. 17, 229–235.

Jaganathan, H., Kinsella, J.M., Ivanisevic, A., 2008. Circular dichroism study of the mechanism of formation of DNA templated nanowires. ChemPhysChem 9, 2203–2206.

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

Kunath, K., Harpe, A.V., Fischer, D., Petersen, H., Bickel, U., Voigt, K., Kissel, T., 2003. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. J. Controlled Release 89, 113–125.

Kurosaki, T., Kitahara, T., Fumoto, S., Nishida, K., Nakamura, J., Niidome, T., Kodama, Y., Nakagawa, H., To, H., Sasaki, H., 2009. Ternary complexes of pDNA, polyethylenimine, and γ-polyglutamic acid for gene delivery systems. Biomaterials 30, 2846–2853.

Lin, J., Zhu, J., Chen, T., Lin, S., Cai, C., Zhang, L., Zhuang, Y., Wang, X.S., 2009. Drug releasing behavior of hybrid micelles containing polypeptide triblock copolymer. Biomaterials 30, 108–117.

Mahato, R.I., 2005. Water insoluble and soluble lipids for gene delivery. Adv. Drug Deliv. Rev. 57, 699–712.

Manocha, B., Margaritis, A., 2008. Production and characterization of γ-polyglutamic acid nanoparticles for controlled anticancer drug release. Crit. Rev. Biotechnol. 28, 83–99.

Merdan, T., Kunath, K., Petersen, H., Bakowsky, U., Voigt, K.H., Kopecek, J., Kissel, T., 2005. PEGylation of poly(ethyleneimine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice. Bioconjugate Chem. 16, 785–792.

Pan, S.R., Wang, Q.M., Yi, W., 2007. Preparation of hydrophilic polyhydroxyalkyl glutamine crosslinked films and its biodegradability. J. Biomater. Appl. 22, 181–192.

Park, T.G., Jeong, J.H., Kim, S.W., 2006. Current status of polymeric gene delivery systems. Adv. Drug Deliv. Rev. 58, 467–486.

Petersen, H., Fechner, P.M., Martin, A.L., Kunath, K., Stolnik, S., Roberts, C.J., Fischer, D., Davies, M.C., Kissel, T., 2002. Polyethyleneimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. Bioconjugate Chem. 13, 845–854.

Pfeifer, A., Verma, I.M., 2001. Gene therapy: promises and problems. Annu. Rev. Genomics Hum. Genet. 2, 177–211.

Rogošić, M., Mencer, H.J., Gomzi, Z., 1996. Polydispersity index and molecular weight distributions of polymers. Eur. Polym. J. 32, 1337–1344.

Shen, Y., Deng, J., Luo, X., Zhang, X., Zeng, X., Feng, M., Pan, S.R., 2009. Synthesis and characterization of sterically stabilized polyelectrolyte using isophorone diisocyanate as the coupling reagent. J. Biomater. Sci., Polym. Ed. 20, 1217–1233.

Tang, G.P., Zeng, J.M., Gao, S.J., Ma, Y.X., Shi, L., Li, Y., Too, H.-P., Wang, S., 2003. Polyethylene glycol modified polyethylenimine for improved CNS gene transfer: effects of PEGylation extent. Biomaterials 24, 2351–2362.

Wang, Y., Chang, Y.C., 2003. Synthesis and conformation transition of surface-tethered polypeptide: poly(L-lysine). Macromolecules 36, 6511–6518.

Wen, Y., Pan, S., Luo, X., Zhang, X., Zhang, W., Feng, M., 2009. A biodegradable low molecular weight polyethylenimine derivative as low toxicity and efficient gene vector. Bioconjugate Chem. 20, 322–332.

Yang, J.P., Huang, L., 1997. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. Gene Ther. 4, 950–960.

Zubkov, V.A., Birshtein, T.M., Milevskaya, I.S., Volkenstein, M.V., 1971. Circular dichroism calculation for random-coil polypeptide chains. Biopolymers 10, 2051–2061.

Zhan, X., Pan, S.R., Hu, H.M., Wu, G.F., Feng, M., Zhang, W., Luo, X., 2008. Poly(ethylene glycol)-block-polyethylenimine copolymers as carriers for gene delivery: effects of PEG molecular weight and PEGylation degree. J. Biomed. Mater. Res. Part A 84, 795–804.